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Mitochondrial RNA metabolism during mitochondriogenesis in yeast

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Abstract. Mitochondrial transcription has been studied as a function of mitochondriogenesis in yeast cells. Two systems have been used: synchronously growing cells and cells subjected to glucose repression followed by derepression. Maximal RNA synthesis has been found in the S phase of the cell cycle and during the 'repressed' phase in asynchronous cells. Activities of RNA polymerase, poly A polymerase and incorporation of [^{32}P]-into RNA *in vitro* are maximal at the same period. Gel analysis reveals the presence of some high molecular weight RNA species which are likely to be precursors. When chase experiments are carried out in the presence of excess glucose, the high molecular weight species remain unaffected, suggesting that RNA processing may be an important site of action of glucose repression.

Keywords. Mitochondrial RNA; mitochondrial RNA polymerase; glucose repression–derepression; synchronous cultures.

Introduction

Evidence that during mitochondriogenesis in yeast, the assembly of the electron transport chain components occurs in a sequential order rather than in a concerted fashion, have been obtained by the following observations reported from this laboratory. (i) By the judicious use of the differentially acting antibiotics, cycloheximide (CHI) and chloramphenicol (CAP), it was shown that the mitochondrially and cytosolically synthesised protein components can be accumulated independent of each other and then reconstituted *in vitro* (Chandrasekaran *et al.*, 1980). (ii) A second addition of glucose at the onset of derepression of enzyme activities keeps the mitochondrial functions in the repressed state, but the levels of cytosolically synthesised mitochondrial components remain at the same level or even enhanced (Chandrasekaran *et al.*, 1978). (iii) Using immunoprecipitation techniques, it has been shown that the mitochondrially synthesised subunits of cytochrome C oxidase follow a partial order (Chandrasekaran *et al.*, 1980). (iv) That the two protein synthesising systems do not act in concert was also shown in synchronous cultures in which the cytosolically synthesised proteins F_1-ATPase and 3 subunits of cytochrome oxidase accumulated in the cytosol during the S-phase and were sequentially integrated into the mitochondrial membrane at the G_2 phase (Somasundaram and Jayaraman, 1981a, b). In this context it was of relevance to investigate the mitochondrial transcriptional

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Abbreviations used: CHI, Cycloheximide; CAP, chloramphenicol; TCA, tricarboxylic acid.

events which should occur prior to mitochondriogenesis, as defined by the expression of the electron transport enzyme activities.

Mitochondria synthesise all their RNA and no import occurs into the organelle (Borst and Grivell, 1978). Mitochondrial transcription *per se* is fairly well-documented (Christianson *et al.*, 1982; Kelly and Phillips, 1983). Neupert and his group (Neupert and Rucker, 1976; Neupert, 1977) have proposed a nuclear trigger to initiate mitochondriogenesis and molecules like cAMP, hemin and GDP have been variously proposed as likely candidates (Chandrakumar and Padmanabhan, 1980; Ohashi and Schatz, 1980; Somasundaram *et al.*, 1980). Initiation of transcription should be the first event in mitochondriogenesis and consequently, establishing the stage of growth of yeast cells at which this process occurs, would be essential to locate and identify this 'trigger molecule'. It was with this approach we have studied the mitochondrial RNA metabolism in yeast cells during mitochondriogenesis.

Materials and methods

Culture and growth conditions

Saccharomyces cerevisiae NCIM 3095, a diploid strain, obtained from the National Collection of Industrial Microorganisms, Poona, was used in these studies and stock cultures were maintained in nutrient agar slants. Cells were grown at $28 \pm 1°C$ for 10–12 h with aeration in a liquid medium containing glucose (1 %), yeast extract (0·4 %), KH_2PO_4 (0·3 %), $MgSO_4 \cdot 7H_2O$ (0·05 %), $CaCl_2$ (0·04 %) and $(NH_4)_2SO_4$ (0·04 %). The harvested cells were used in experiments involving synchronous cultures or glucose repression/derepression system.

Cells were synchronously grown following the method of Mitchison and Vincent (1965) and the behaviour of these cultures were same as described in earlier communications (Somasundaram and Jayaraman, 1981a, b). Good synchrony was obtained upto 3–4 generations. In our studies we have used the second generation cells.

Conditions of glucose repression/derepression have been described earlier in detail (Jayaraman *et al.*, 1966). Briefly, the harvested cells were resuspended in the same medium at a concentration of about 4 mg wet wt cells/ml medium and allowed to grow at 28°C with shaking. Under these conditions, during the first $2\frac{1}{2}$ h (designated as repression phase), the respiratory rate as well as the levels of several mitochondrial enzyme activities and components decrease 4–5 fold, whereas the cells grow exponentially. After $2\frac{1}{2}$ h, the cells enter the diauxic growth (glucose in the medium having been exhausted) and exhibit a rapid increase in various mitochondrial parameters (designated derepression phase). Having established in preliminary studies that cell number and turbidity at 600 nm were linear, for routine experiments, turbidity was followed.

Respiratory activity was monitored using Clark type oxygen electrode (Model YSI 4004) (Borst and Grivell, 1978). Protein was assayed by the method of Lowry *et al.* (1951). RNA was estimated by measuring the absorbance at 260 nm and 280 nm (1 $A_{260\,nm} = 40\,\mu g$ RNA) (Jayaraman, 1981). Mitochondrial RNA polymerase was prepared according to the method of Scragg (1974) by solubilisation from isolated mitochondria and was assayed following the method of Kuntzel and Schafer (1971).

Poly A polymerase was partially purified by the method of Rose *et al.* (1976) and assayed following the method of Jeyaraj *et al.* (1982).

Isolation of mitochondrial RNA

Mitochondria were prepared according to the method of Jayaraman *et al.* (1966) in an isolation buffer containing 0·25 M sucrose, 10 mM Tris-HCl, pH 7·4, 3 mM EDTA, 4 μg/ml heparin and 0·1 % diethyl pyrocarbonate. The mitochondria were lysed in a solution containing 0·1 M NaCl, 0·1 M Tris-HCl, pH 9·0, 1 mM EDTA, 0·5 % sodium dodecyl sulphate, 4 μg/ml heparin and 0·1 % diethyl pyrocarbonate. RNA was isolated after deproteinisation with phenol/chloroform/isoamyl alcohol (50/50/l), by precipitation with ethanol from the aqueous phase. The RNA was washed with 2 M LiCl and stored in 10 mM Tris-HCl, pH 8·0 (containing 1 mM EDTA) at $-20°$C (Suzuki and Brown, 1972).

Radiolabelling experiments

In synchronous cultures: Carrier free [^{32}P]-phosphate and [^{14}C]-chlorella hydrolysate (sp. act. 20 mCi/matomC) as the case may be, were added to the cultures at the beginning of each phase, G_1, S, G_2 and M phases, respectively, and cells harvested at the end of the phase. Earlier experiments revealed that each phase was of 30 min duration (Somasundaram and Jayaraman, 1981a).

Under glucose repression/derepression conditions

In these experiments, the cells were exposed to [^{32}P]-phosphate for 1 h duration at various time intervals *viz.* 0–1 h, 1–2 h and so on.

In chase experiments, cells were labelled from 1–2 h and the radioactive label was chased by the addition of 0·01 M cold phosphate for a further period of 1 h. During the chase period additions were made as described under 'results'.

In vitro labelling

Synthesis of RNA by isolated mitochondria was followed according to the method of Newman and Martin (1982) with minor modifications.

In all cases, the RNA was precipitated with cold 10 % trichloroacetic acid (TCA), followed by filtration through glass fibre filters (GF/A Whatman, 2·4 cm) and extensive washing with 5 % TCA and ethanol. The filters were dried and radioactivity determined using a toluene based scintillation fluid (Jeyaraj *et al.*, 1982).

Gel electrophoresis of RNA

Electrophoresis of RNAs was carried out in 2 % acrylamide, 0·5 % agarose composite gels (Peacock and Dingman, 1968). Ethidium bromide stained gels were photographed under a short range ultraviolet source of 254 nm. The gels were dried, sliced into 3 mm bits and counted after hydrolysis (Jeyaraj *et al.*, 1982). The *S* values were standardised by using yeast cytosolic rRNA and tRNA markers.

Results

RNA labelling in synchronous cultures

Figure 1 shows the pattern of synchrony, oxygen uptake, [14C]-amino acid incorporation into mitochondrial proteins, and [32P]-incorporation into mitochondrial RNA. It is clearly seen that mitochondrial RNA synthesis is most active in the S-phase, (table 1) whereas incorporation of amino acids into mitochondrial proteins and respiratory activities were maximal at G_2 phase (Somasundaram and Jayaraman, 1981a, b).

Analysis of labelled RNA species

The RNA samples labelled at various phases were analysed in agarose–acrylamide composite gels (figure 2). At G_1 phase, there is synthesis of very few species of RNA and the label is predominantly observed in a high molecular weight species (about 45S) in addition to a species slightly less than 14S. Although 14S rRNA is labelled, interestingly, 21S ribosomal RNA and 4S tRNAs are not labelled. The profile changes drastically during the S phase. In addition to the ribosomal RNAs and tRNAs, labelling is observed in the 'messenger region'. Further, in the high molecular weight region (35S–45S), 3 prominent peaks are seen. At the G_2 phase, the high molecular weight species have almost disappeared and labelling was prominent in the 'messenger region'. In the M phase, there was practically no labelling of any species.
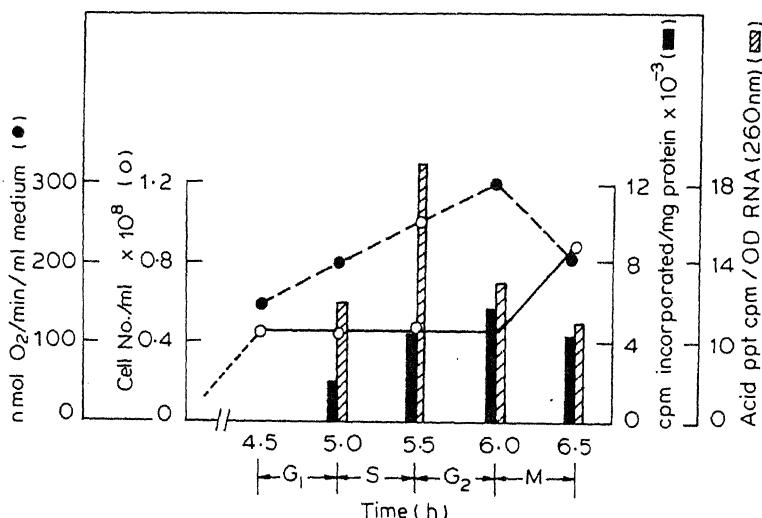
Figure 1. Pattern of synchrony, oxygen uptake, mitochondrial protein and RNA synthesis in synchronous cultures of yeast.

Synchronous cultures of yeast grown to two cell cycle divisions were labelled with either [14C]-chlorella hydrolysate (2 μCi/ml medium) or [32P]-phosphate (1 mCi/100 ml medium). Oxygen uptake, (●), cell growth (O), incorporation of [14C]-chlorella hydrolysate into mitochondrial proteins (▨) and [32P]-phosphate into RNA were monitored (■) at intervals of 30 min.

Table 1. *In vivo* labelling of RNA with [^{32}P]-phosphate in synchronous cultures of yeast.

Labelled RNA from synchronous cultures of yeast grown in the presence of [^{32}P]-phosphate (1 mCi/100 ml medium) was extracted and the acid precipitable radioactivity determined. During nalidixic acid treatment, the antibiotic (50 μg/ml) was added along with the label.

Stages of growth	Acid precipitable radioactivity (cpm/mg RNA)
G$_1$	$3\cdot0 \times 10^5$
S	$4\cdot7 \times 10^5$
G$_2$	$3\cdot4 \times 10^5$
M	$2\cdot7 \times 10^5$
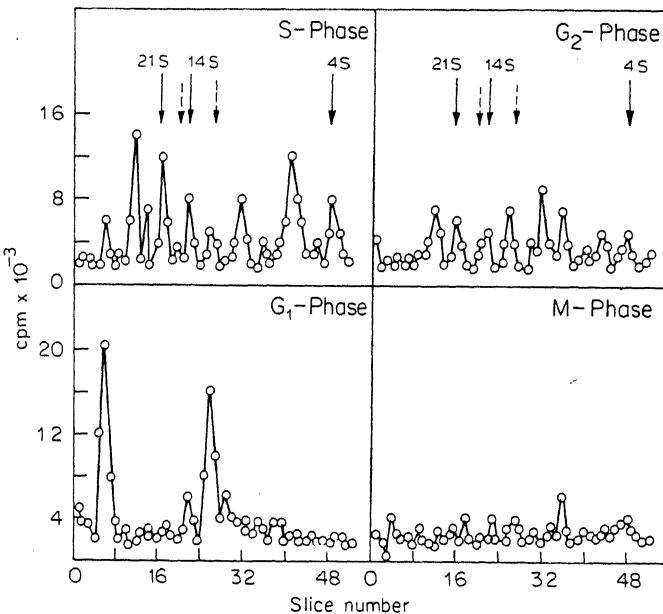
Figure 2. Analysis of labelled RNA species in synchronous cultures of yeast.
Labelled mitochondrial RNA (1 A$_{260\,nm}$/slot) was analysed on a 2% acrylamide–0·5% agarose composite gel. At the end of the run the gel was sliced into 0·3 cm bits and processed for measuring radioactivity.

Studies using glucose repression/derepression conditions

Since working with synchronous cultures on a large scale was difficult and also since the glucose repression/derepression system simulated the synchronous growth conditions with respect to mitochondriogenesis (Jayaraman *et al.*, 1975), we resorted to the latter system for further studies. The various changes that take place in this system as a function of time have been described in earlier communications (Jayaraman *et al.*, 1966, 1975).

[^{32}P]-Labelling of mitochondrial RNA at different time intervals was studied (table 2). Maximum incorporation into RNA was observed between 1 and 2 h. RNA species labelled at different times were analysed in the gel (figure 3). The following features were noticed.

(i)   During the first 1 h, a few species mainly in the region between 4S and 14S are labelled. The 14S RNA is labelled while 21S and 4S RNAs are not. This resembles the situation obtained in the G_1 phase cells, except that the high molecular weight RNA is not seen.

(ii) Between 1 and 2 h, there is a burst of transcriptional activity. The interesting point to note is the presence of atleast 4 radioactive peaks above 21S, ranging between 35S–45S. This resembles the pattern obtained in the S phase of synchronous cultures.

(iii) Between 2 and 3 h, the intensity of labelling of all species has decreased. Only one peak in the region above 21S was observed.

(iv) Between 3 and 5 h, there is very little synthesis of RNA.

Effect of second addition of glucose

In the next series of experiments, labelling was carried out in a similar way as shown in table 3, but at each time point a second addition of glucose was made to bring the final concentration to 1 %. There was more than 50 % inhibition in the period between 1–2 h, showing that glucose concentration is a factor in mitochondrial transcription (results not given).

Table 2. Changes in the total mitochondrial RNA and poly(A) RNA levels during repression/derepression.

Labelled RNA from different time hour mitochondria were extracted and the acid precipitable radioactivity and the radioactivity bound to poly(U) millipore filters determined (see methods).

Time of growth	RNA yield A_{260}/50 ml culture	Acid precipitable cpm/ incorporated/mg RNA	cpm bound to poly(U) filter/mg RNA	% poly(U) RNA
10 min	1·12	$0·5 \times 10^5$	$0·02 \times 10^5$	3·7
1 h	1·80	$4·1 \times 10^5$	$0·21 \times 10^5$	5·8
2 h	2·40	$12·6 \times 10^5$	$1·80 \times 10^5$	14·9
3 h	3·52	$5·4 \times 10^5$	$0·57 \times 10^5$	10·6
4 h	3·60	$1·7 \times 10^5$	$0·13 \times 10^5$	7·5
5 h	3·72	$0·9 \times 10^5$	$0·03 \times 10^5$	3·8
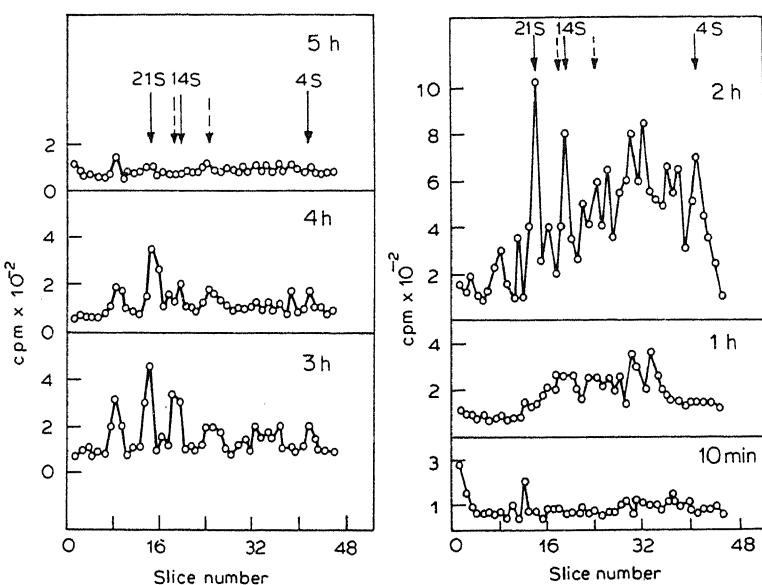
Figure 3. Analysis of labelled mitochondrial RNA in cells undergoing glucose repression/derepression.

Labelled mitochondrial RNA isolated from different time points in cells undergoing repression/derepression was analysed in an agarose-acrylamide composite gel, the gel was sliced and processed for measuring radioactivity.

Table 3. Chase of the 2 h labelled mitochondrial RNA in the presence of glucose and antibiotics.

Cells labelled from 1–2 h were chased for 1 h in the presence of excess of cold phosphate containing either 1% glucose, nalidixic acid (50 μg/ml), chloramphenicol (5 mg/ml) or cycloheximide (100 μg/ml).

Time of chase	Acid precipitable cpm incorporated/mg RNA
0 h chase	$11{\cdot}4 \times 10^5$
1 h chase:	
control	$9{\cdot}3 \times 10^5$
+ Glucose	$7{\cdot}7 \times 10^5$
+ Cycloheximide	$8{\cdot}6 \times 10^5$
+ Chloramphenicol	$7{\cdot}5 \times 10^5$
+ Nalidixic acid	$6{\cdot}1 \times 10^5$

Chase experiments

In order to assess the turnover of the RNAs synthesised between 1 and 2 h, a few chase experiments were carried out. Cells were labelled between 1 and 2 h and at that time 0·01 M cold phosphate was added to chase the label. After 1 h chase, cells were harvested and mitochondrial RNA isolated and analysed. The chase also was carried

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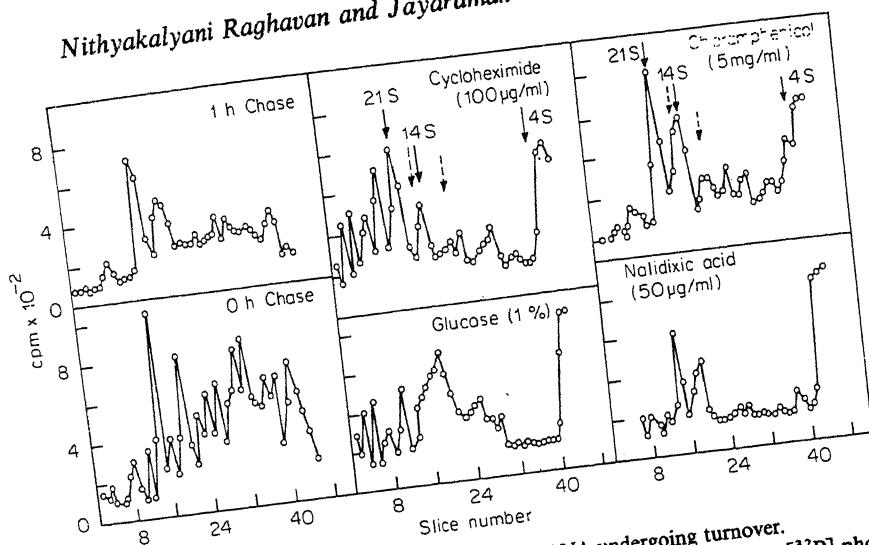
Figure 4. Analysis of labelled mitochondrial RNA undergoing turnover. Cells undergoing repression/derepression were labelled from 1–2 h with [^{32}P]-phosphate and chased for the next 1 h in the presence of excess 0·01 M phosphate. The chase was also carried out in the presence of inhibitors as indicated. The mitochondrial RNA was extracted and analysed in an agarose-acrylamide composite gel.

out in the presence of glucose (1% final concentration) and the protein synthetic inhibitors, cycloheximide and chloramphenicol. Results are given in table 3. The gel profiles of the RNA species after chase gave some interesting results (figure 4). After 1 h chase, the high molecular weight species (barring one peak) have disappeared and there is considerable reduction in 7S–11S region, whereas the 21S, 14S and 4S species are more stable.

Interesting changes are observed when the chase was carried out in the presence of glucose. There is an accumulation of species in the high molecular weight range (about 14S, infact) while the small molecular weight species have decreased label. A similar situation is obtained in the presence of cycloheximide. In the presence of chloramphenicol, labelling in the high molecular weight weight range declines, while labelled 4S–11S species persist.

RNA synthesis by isolated mitochondria

The results of experiments in which [^{32}P]-labelling of RNA by isolated mitochondria are given in table 4. Conforming to the pattern obtained in *in vivo* labelling experiments, mitochondria isolated from cells at 2 h of growth show maximal incorporation. The incorporation is insensitive to chloramphenicol and oligomycin but inorganic pyrophosphate and a mixture of nalidixic acid and actinomycin D cause drastic inhibition (table 5).

Mitochondrial RNA polymerase activity

The RNA polymerase activity of mitochondria isolated from cells at different times were also analysed and the results given in table 6 again show the highest activity at 2 h.

Table 4. *In vitro* labelling of mitochondrial RNA.

Isolated mitochondria from cells undergoing repression/derepression at different time periods of growth were labelled with 100 μCi/ml of [^{32}P]-phosphate as described in 'methods' and the RNA extracted and analysed.

Time of growth	Acid precipitable cpm incorporated/mg RNA
0	0.53×10^5
1	1.57×10^5
2	9.50×10^5
3	2.60×10^5
4	1.80×10^5
5	0.90×10^5

Table 5. Effect of inhibitors on the *in vitro* labelling of mitochondrial RNA.

Isolated mitochondria from cells harvested at 2 h were labelled *in vitro* with [^{32}P] in the presence of various inhibitors like nalidixic acid (50 μg/ml) + actinomycin D (5 μg/ml), chloramphenicol (5 mg/ml), oligomycin (50 μg/ml), inorganic pyrophosphate (5 mg/ml) and the incorporation determined.

Sample	Acid precipitable cpm incorporated/mg RNA	% inhibition
Control	41.7×10^5	
+ Nalidixic acid and Actinomycin D	5.2×10^5	87.5
+ Chloramphenicol	31.1×10^5	25.4
+ Oligomycin	40.7×10^5	2.4
+ Inorganic pyrophosphate	3.1×10^5	92.6

Table 6. Mitochondrial RNA polymerase activity as a function of repression/derepression.

Mitochondria were isolated from cells grown to different time periods. The mitochondrial RNA polymerase was extracted and assayed using [^{3}H]-ATP, as described under 'methods'. Nalidixic acid was used at a concentration of 50 μg/ml

Time	Acid precipitable cpm incorporated/mg protein/h	+ nalidixic acid	% inhibition
10 min	1.6×10^5	7.5×10^4	53.1
1 h	2.04×10^5	6.2×10^4	69.6
2 h	2.79×10^5	1.44×10^4	94.8
3 h	1.74×10^5	1.22×10^4	93.0
4 h	1.34×10^5	—	—
5 h	1.13×10^5	9.1×10^4	19.5

Table 7. Mitochondrial poly (A) polymerase activity during repression–derepression.

DEAE cellulose-purified mitochondrial poly (A) polymerase from different time hour mitochondria was analysed as described in 'methods' and the activity defined as p mol ATP incorporated/mg protein/h.

Time	[^{3}H]-ATP incorporated/mg protein/h	
	cpm	p mol
0	0.71×10^5	40.0
1	1.50×10^5	76.5
2	5.40×10^5	274.6
3	2.60×10^5	130.6
4	2.70×10^5	135.4
5	2.20×10^5	111.2

Inclusion of nalidixic acid in the assay system at 50 μg/ml concentration shows more than 90 % inhibition in the case of 2 h and 3 h mitochondria, whereas it is less at other times. The enzyme activity is inhibited when cells are exposed to higher concentration of glucose before harvesting (results not given).

Poly A adenylation and poly A polymerase activity

There exists a controversy about the existence of poly A tails in yeast mitochondrial RNA (Groot *et al.*, 1974; Aujane and Freeman, 1976). However in our strain, we have found that mitochondrial RNA isolated from cells harvested at 2 h, has 14.9 % poly A content and at 3 h it drops to 10.6 % (table 2). Poly (A)polymerase activities were also monitored (table 7) and once again maximal activity of 254 p mol [^{3}H]-ATP incorporated/mg protein/h is obtained at 2 h.

Discussion

'Glucose repression' is a terminology used with reference to the expression of mitochondrial energy transducing functions. Baldacci and Zennaro (1982) have reported on the mitochondrial RNA metabolism of 'repressed' yeast cells (*i.e.* cells grown to mid exponential phase in 15 % glucose medium) and compared that with that of 'derepressed cells' (*i.e.* cells grown in the presence of glycerol).

The systems we have used in these studies offer us the facility to follow the mitochondrial transcriptional pattern as a function of the stages of mitochondriogenesis. Using these systems, we have restricted ourselves to investigating the total transcriptional activity of the mitochondria at various stages, and looking at the newly synthesised RNA species in a qualitative way. In this limited context, we have designated any RNA species above 21S as putative 'precursors' and the species appearing between 4S and 14S as the 'messenger region'. The translatability of the RNAs, isolated at various times and conditions, has been studied in rabbit cell-free reticulocyte lysate system and the pattern of [^{14}C]-amino acid incorporation into TCA

precipitable material in such experiments, follows closely the appearance and disappearance of species in the messenger region (Nithyakalyani Raghavan and J. Jayaraman, unpublished data).

In synchronous cultures, maximal mitochondrial transcription occurs during the S phase, followed by translation and expression of electron transport enzyme activities in the subsequent phase whereas G_1 and M phases are silent. It is interesting to note that a high molecular weight RNA species is labelled at this phase. When the cells enter the S phase, the mitochondrial transcriptional machinery is very active, synthesising several species from 45S–4S. In the G_1 phase, labelling is mainly found in the 'messenger region' apart from rRNAs and tRNAs.

A somewhat analogous situation is obtained in the glucose repression–derepression condition also. Maximal incorporation of $[^{32}P]$-phosphate into RNA occurs between 1 and 2 h of growth and the gel profile shows a remarkable similarity to the RNA obtained from S-phase cells. Between 2 and 3 h labelling is mainly in the 'messenger region' with considerable reduction in high molecular weight species representing the RNA processing period. Assay of transcription-associated enzyme activities, RNA polymerase and poly A polymerase, as well as *in vitro* incorporation of $[^{32}P]$-phosphate into RNA, all substantiate the fact that between 1–2 h [which will correspond to the mid-exponential phase cells grown in higher concentration of glucose by other workers (Hendler *et al.*, 1975) and which is defined as 'repressed phase'] has the maximal mitochondrial transcriptional activity.

We deduce from these experiments that late G_1 or early S phase cultures in synchronous growth conditions, or after 1 h growth under the repression–derepression conditions, would be the time point to try and locate the nuclear trigger, proposed by Neupert's group (Neupert and Rucker, 1976; Neupert, 1977). The effect of cAMP and hemin, suggested as possible trigger molecules (Somasundaram *et al.*, 1980; Chandrakumar and Padmanabhan, 1980) was tried in our system but found a small but overall increase in transcription (1·2 fold in the case of cAMP and 4 fold in the case of hemin, table 8), but the gel patterns remained the same (data not presented). Only hybridisation studies using mitochondrial DNA probes will answer the question as to whether any specific species are induced. Increasing the glucose concentration in the medium during the period of active growth-diminished transcription in accordance with the results of Baldacci and Zennaro (1982). Interestingly, the apparently nuclear-coded RNA polymerase activity was also inhibited.

Table 8. Effect of hemin and cyclic AMP addition on the synthesis of mitochondrial RNA.

Cells undergoing repression/derepression were labelled from 0–1 h with $[^{32}P]$-phosphate (2 mCi/200 ml medium) in the presence of either hemin (2·5 μg/ml) or cyclic AMP (5 mM)

Sample	Acid precipitable cpm incorporation/gm RNA	Stimulation
Control	$1\cdot4 \times 10^5$	
+hemin	$5\cdot0 \times 10^5$	257%
+cAMP	$1\cdot6 \times 10^5$	14·3%

The chase experiments gave some interesting data. In these, cells were labelled between 1 and 2 h and then label chased for a further 1 h. In the control cells, the high molecular weight species disappeared as also most species in the messenger region. Only the rRNAs and tRNAs retained the label to a large extent. But if excess glucose was present during the chase, there was a net accumulation of high molecular weight species. Baldacci and Zennaro (1982) have also observed the accumulation of a high molecular weight RNA species, faulty maturation of *cob* precursor RNA and a reduction of *oli* 1 and *oxi* 1 mRNAs in repressed cells.

This could indicate that RNA processing may be a crucial step in the manifestation of 'glucose effect' on mitochondriogenesis.

Chase experiments in the presence of cycloheximide showed accumulation of high molecular weight RNA species, testifying to the cytosolic origin of the processing enzymes. Chloramphenicol did not show such an effect.

Precise hybridisation studies using mitochondrial DNA probes, which are under way, will answer some of the questions raised.

Acknowledgements

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Guggulsterone induced changes in the levels of biogenic monoamines and dopamine β-hydroxylase activity of rat tissues*

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Abstract. The effect of lipid lowering agent, guggulsterone, the purified fraction of guggulipid, on biogenic monoamine levels and dopamine β-hydroxylase activity of rat brain and heart has been studied. Administration of guggulsterone caused inhibition of brain dopamine β-hydroxylase activity with marked stimulation in heart both *in vivo* and *in vitro*. The levels of catecholamines were similarily affected. The contents of serotonin and histamine were found to be enhanced in brain and decreased in heart. Alterations in biogenic amines and dopamine β-hydroxylase activity may be one of the possible mechanisms for the antilipaemic effect of the compound.

Keywords. Guggulsterone; guggulipid; *Commiphora mukul*; hypolipidaemic; dopamine β-hydroxylase; catecholamine.

Introduction

It was reported earlier that guggul resin and its fractions showed hypocholesterolemic activity (Kapoor and Nityanand, 1971, 1976; Nityanand and Kapoor, 1971, 1973, 1975). In view of the important role of biogenic amines in hyperlipidaemia and atherosclerosis (Nityanand, 1967; Kapoor and Nityanand, 1969; Bhattacharya *et al.*, 1974; Srivastava *et al.*, 1984c), the effect of lipid lowering agent, garlic oil and guggulipid (Srivastava *et al.*, 1984a, b) were studied. These showed marked enhancement in biosynthesis of catecholamines in normal and hyperlipaemic animals. Patil *et al.* (1972) reported on the steroidal constituents of guggul resin from *Commiphora mukul* and isolated pure guggulsterone 'Z' and 'E' isomers, for the first time from petroleum ether fractions and established their chemical structures. With a view to investigate the effect of more purified form of guggul resin (guggulipid), the effect of guggulsterone has been studied on the levels of biogenic monoamines and dopamine β-hydroxylase (DBH) activity of rat brain and heart.

Materials and methods

Adult male rats of Charles Foster strain (100–150 g) were obtained from Institute animal colony. They were maintained on a standard animal house diet. Experiments

were designed to investigate the effect of guggulsterones on DBH activity and biogenic amine levels. The *in vitro* effect of guggulsterones on DBH activity of brain and heart was studied using different concentrations as reported earlier (Srivastava and Kapoor, 1979a). A stock solution of guggulsterones (10 mM) was prepared in propylene glycol and diluted accordingly to give final concentrations ranging from 0·01–1·0 mM. In the reaction mixtures of control tubes the same volume of propylene glycol was added. The acute (*in vivo*) effect of guggulsterone on DBH activity of brain and heart was studied by injecting guggulsterone (100 mg/kg body weight, dissolved in propylene-glycol and gum acacia) to 8 rats intraperitoneally. Controls were given propylene-glycol and gum acacia. Rats were killed by cervical dislocation 6 h after the single dose. The chronic effect of guggulsterone on DBH activity and biogenic amine levels of brain and heart was also studied. Rats were divided into 3 groups of 8 rats each. Guggulsterones (50 mg/kg body weight, dissolved in propylene-glycol and water in 1:4 ratio) were injected intraperitoneally to the rats of the second and third groups for 5 days and 10 days respectively at 24 h intervals. Controls (first group) were given propylene-glycol and water in 1:4 ratio. Rats were killed 2 h after the last dose. Biogenic amines were estimated according to the procedure described earlier (Srivastava and Kapoor, 1979b, 1983a, b).

Results

In vitro effect of guggulsterones on rat brain and heart DBH activity (table 1) was studied showing that the brain enzyme activity was inhibited progressively with maximum inhibition of about 50% at 1 mM concentration. The heart DBH activity was stimulated by about 73% at 1 mM concentration. On acute treatment (*in vivo*) of guggulsterones in rats, no significant effects on DBH activity of brain and heart were observed.

Table 2 shows changes in catecholamine biosynthesis of rat brain and heart by chronic treatment of guggulsterones. In brain, DBH activity was reduced by 21·5% after 5 days treatment and by 42·4% after 10 days treatment. Heart DBH activity showed 37% and 51·3% stimulation after 5 and 10 days treatment respectively. The levels of catecholamines and DBH activity were regulated in a coordinate fashion by the

Table 1. *In vitro* effect of guggulsterone on dopamine β-hydroxylase activity of rat brain and heart.

Guggulsterone (mM)	Specific activity* of DBH	
	Brain	Heart
0·00	52·6±3·2	45·2±2·4
0·01	37·6±2·1[a]	49·9±2·8
0·10	33·8±2·5[b]	65·8±3·5[b]
1·00	28·4±1·6[b]	78·2±4·4[b]

* n mol octopamine formed per mg protein per 30 min.
Values are mean ±S.E.M. of 8 observations.
[a]$P < 0.01$; [b]$P < 0.001$ with respect to control.

Table 2. Effect of guggulsterone treatment on catecholamine level and DBH activity of rat brain and heart.

| Tissue | Treatment | Catecholamine level (ng/g wet tissue) | | DBH specific activity* |
		Dopamine	NE	
Brain	Control	584 ± 35.4	290 ± 15.5	54.2 ± 3.3
	5 days	554 ± 26.6	252 ± 11.5^a	42.5 ± 2.1^b
	10 days	504 ± 23.1^a	232 ± 13.9^b	31.2 ± 1.2^c
Heart	Control	48 ± 2.8	242 ± 8.2	43.8 ± 2.4
	5 days	82 ± 4.6^c	269 ± 11.6	60.0 ± 4.8^c
	10 days	132 ± 9.8^c	379 ± 12.4^b	66.3 ± 4.5^c

* n mol octopamine formed per mg protein per 30 min.
Values are mean $\pm$ S.E.M. of 8 observations.
$^aP < 0.05;$ $^bP < 0.01;$ $^cP < 0.001$ with respect to control.

guggulsterones. In brain, the levels decreased continuously on increasing the duration of treatment while in the heart catecholamines increased progressively. However, the changes in the levels of heart catecholamines were more marked than in brain.

Alterations in serotonin (5-HT) and histamine levels of brain and heart by guggulsterones treatment are represented in table 3. A progressive increase was observed in the brain levels of 5-HT and histamine showing 25 and 41 % increase in 5-HT level and 21 and 36·6 % increase in histamine levels after 5 and 10 days treatment respectively. Heart 5-HT and histamine levels were found to be reduced marginally showing only 24 and 27·5 % decline in 5-HT level and 5 and 13 % decline in histamine level after 5 and 10 days treatment respectively.

Table 3. Effect of guggulsterone treatment on 5-HT and histamine level of rat brain and heart.

Tissue	Treatment	5-HT (ng/g wet tissue)	Histamine (ng/g wet tissue)
Brain	Control	478 ± 19.3	153 ± 9.3
	5 days	599 ± 26.8	185 ± 9.8
	10 days	673 ± 29.2^a	209 ± 14.1
Heart	Control	330 ± 11.2	741 ± 19.9
	5 days	251 ± 14.5	704 ± 12.5
	10 days	239 ± 11.3^a	644 ± 15.7

All values are mean $\pm$ S.E.M. of 8 observations.
$P < 0.05$ with respect to control.
$^aP < 0.01$ with respect to control.

Discussion

In vitro studies revealed that guggulsterones cause inhibition of brain DBH activity with a marked stimulation in the heart activity. The changes in the enzyme activity may be due to the allosteric alterations or conformational changes in the enzyme molecule by guggulsterone. No significant effect was observed on the enzyme activities of brain and heart by acute *in vivo* treatment of guggulsterones which may be due to unavailability of the drug to the tissue enzyme system in such a short duration of 6 h. On chronic treatment of guggulsterones, marked alterations were observed in biogenic monoamine levels and DBH activity of brain and heart. In addition, the implication of the enzymes, monoamineoxidase and catechol-*o*-methyltransferase, in the regulation of catecholamine levels cannot be ruled out. Our results are in close agreement with those reported for one of the most popular β-adrenergic blocking agent, propanolol, showing a decline in catecholamine biosynthesis in different parts of rat brain (Srivastava and Kapoor, 1983a) and a progressive increase in the case of heart (Srivastava and Kapoor, 1983b). Guggulsterones have also been observed to have β-adrenergic blocking activity at a high dose (unpublished data). However, it is still not clear whether the changes in catecholamine biosynthesis by β-adrenergic blockers are due to their β-blocking activity.

The increased levels of norepinephrine (NE) in the heart and 5-HT in brain can also be correlated with those reported for pyridinol carbamate (Berkowitz *et al.*, 1973). The present study revealed that lipid lowering drugs induce changes in biogenic amine levels and DBH activity and therefore can be evaluated for the treatment of cardiovascular disorders.

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Biochemical lesions in liver and kidney after caecal amoebiasis in rats by *Entamoeba histolytica* and their reversal by antiamoebic drugs

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Abstract. Levels of lipid peroxides in rat caecum, blood, liver and kidney and the capacity of tissue homogenates to form lipid peroxides *in vitro* was enhanced after caecal amoebiasis in rats produced by *Entamoeba histolytica* (IB-1). The activity of hepatic drug-metabolizing enzymes in post-mitochondrial fraction and the cytochrome P_{450} contents in microsomal fraction decreased significantly, while lysosomal enzymes such as acid phosphatase, acid ribonuclease and cathepsin B showed an increase in the liver homogenates of infected animals. These changes were reversed following treatment with the antiamoebic drug, metronidazole.

Keywords. Lipid peroxidation; drug-metabolizing and lysosomal enzymes; cellular injury.

Introduction

It has been reported that intracaecal inoculation of rats with a virulent strain of *Entamoeba histolytica* produces lesion and damage to the wall of caecum (Dutta and Srivastava, 1974). It is not known whether biochemical and histological lesions produced in caecum during amoebiasis, cause secondary damage to other organs like liver and kidney. In view of the reports that prolonged caecal amoebiasis may cause hepatic amoebiasis (Gill *et al.*, 1983a, b), it is imperative that biochemical changes in other tissues are also studied during caecal amoebiasis. Increased lipid peroxidation is considered one of the indices of cellular injury (Sharma *et al.*, 1972; Sato *et al.*, 1979; Suematsu *et al.*, 1977; Nishigaki *et al.*, 1980) and is known to affect structural and functional integrity of biological membranes (Hiramitsu *et al.*, 1976a, b; Tappel *et al.*, 1963; Kalish and Di Luzio, 1966; Wills, 1971; Wills and Wilkinson, 1966) and has been implicated in the pathology of ascariasis and filariasis (Gevondyan and Gevondyan, 1974). Hence we have studied changes in lipid peroxidation in caecum, liver, kidney, brain and blood at different stages of intestinal amoebic infection in rats, the activities of drug-metabolizing and lysosomal enzymes, and the content of cytochrome P_{450} in liver and reversibility of these changes induced by antiamoebic drug metronidazole. The results obtained suggest that although infection is produced in caecum, secondary

biochemical lesions are produced in liver and kidney, presumably, through increased output of lipid peroxides from infected caecum to blood, and these changes are reversed after administration of the antiamoebic drug, metronidazole.

Materials and methods

Twenty one day old male weanling rats (Druckrey strain) weighing 25–30 g drawn from C.D.R.I. Animal House were fed *ad lib* autoclaved rice diet (15 lbs/sq. inch for 10 min) and had free access to water. Vitamin E (10 mg/kg body weight/day) was also administered orally to all rats till they were sacrificed. The rats were divided into 4 groups after 7 days of this dietary regimen. Three rats were kept in each cage. The rats of group I and II were inoculated intracaecally with 100,000 trophozoites of *E. histolytica* (IB-1) during laprotomy. The methods used for maintaining *E. histolytica* and inoculation procedure in rats were similar to those reported earlier (Krishna Prasad and Bansal, 1982, 1983). After two days of inoculation the rats of group II (infected) and group III (uninfected) were orally administered metronidazole (May and Baker). 0·5 ml of metronidazole dissolved in sterile water (100 mg/kg/day) was given for 5 continuous days. The rats of group I and IV received normal saline and served as control. Three rats from group I and II were killed by decapitation on the 3rd and 7th day after inoculation, while the rats of group III and IV were killed on the 5th and 7th day after dietary regimen of one week. All the rats were starved overnight before killing. The liver, brain, kidney and caecum were excised out immediately and washed with chilled normal saline. Blood was collected from the severed blood vessels of the neck.

Monitoring of infection

Infection was ascertained by microscopic examination of caecal contents for the presence of *E. histolytica* and gross examination of caecal walls for thickening or ulceration, Neal's (1951) method was used for evaluating infection.

Assay of lipid peroxides

Ten per cent (w/v) homogenates of all tissues were prepared in 1·15 % KCl (w/v) using Potter-Elvejhem homogeniser fitted with teflon pestle. Tissue homogenates were incubated in 25 ml Erlenmeyer flasks at 37°C for 2 h in Dubnoff metabolic shaker (120 horizontal strokes per min). Aliquots were withdrawn at 0 h and 2 h for assaying lipid peroxides according to Ohkawa *et al.* (1979). Lipid peroxides in blood was estimated according to Yagi (1976).

Fractionation of liver homogenate

The homogenate was centrifuged at 1000 *g* for 10 min to remove cell debris and the resultant supernatant at 29,000 *g* for 15 min to separate the mitochondrial pellet. The mitochondrial pellet was washed twice by resuspending it in 1·15 % KCl (w/v) and centrifuging at 29,000 *g* for 15 min. All the supernatants were combined and centrifuged at 105,000 *g* for 60 min. The microsomal fraction thus obtained was washed

once by resuspending in 1.15% (v/v) KCl and recentrifuging at 148,000 g for 60 min. All these centifugations were performed at 0–4°C.

Enzyme assay in liver

Drug-metabolizing enzymes aniline hydroxylase (Cochin and Axelrod, 1959) and aminopyrine N-demethylase (Kato and Gillette, 1965) were assayed in post-mitochondrial supernatant. Lysosomal acid phosphatase (Wright *et al.*, 1972), acid ribonuclease (De Duve *et al.*, 1955) and Cathepsin B (Mycek, 1970) were assayed in the unfractionated homogenate without freezing and thawing. Cytochrome P_{450} content was assayed in microsomal fraction according to the method of Omura and Sato (1964).

Results

All the rats following inoculation with *E. histolytica* developed caecal amoebiasis as evidenced by thickening of the caecal wall with ulceration and appearance of mucous in the caecal lumen. The results summarized in table 1 show that the level of lipid peroxides in rat caecum, blood, liver and kidney increased progressively after amoebic infection in the caecum but there was no significant change in the level of lipid peroxides in the brain. The tissue injury in caecal walls during amoebic infection causes increased lipid peroxidation in caecum and there is an increased out put of lipid peroxides from the site of infection to blood. The capacity of the homogenates of caecum, liver and kidney of infected rats to form lipid peroxides during *in vitro* incubation at 37°C was also more than that in the tissues from uninfected rats. It is possible that certain pro-oxidant factor(s) are elaborated during amoebic infection. The results of table 2 show that the activities of drug-metabolizing enzymes aminopyrine N-demethylase and aniline hydroxylase in the post-mitochondrial supernatant and cytochrome P_{450} content of the liver microsomal fraction decreased significantly in the infected group as compared to that of the uninfected one. On the other hand activities of lysosomal enzymes *viz.* acid phosphatase, acid ribonuclease and cathepsin B increased through infection. It is possible that these changes in liver are consequences of increased lipid peroxidation.

After 5 days of treatment of infected rats with metronidazole, thickening and ulceration of caecal walls and mucous contents were reduced to normal levels. The results of table 3 demonstrate that the level of lipid peroxides in blood, caecum, liver and kidney were also restored to normal values. Production of lipid peroxides *in vitro* by liver and caecum homogenates decreased considerably after 5 days of drug-treatment. The results presented in table 3 also show that the activities of aminopyrine N-demethylase, aniline hydroxylase, acid phosphatase acid ribonuclease, cathepsin B and contents of cytochrome P_{450} were similarly restored after treatment with metronidazole. However, when metronidazole was administered to uninfected rats, the activities of these enzymes remained unchanged as compared to those of untreated rats.

Discussion

It is clear from the above results that establishment of amoebic infection causes increase in the level of lipid peroxide *per se* in caecum, liver and kidney and the capacity of

Table 1. Lipid peroxidation in caecum, liver and kidney.

		Uninfected			Infected		
	Days after infection	0h	2h	Lipid[a] peroxides in vitro	0h	2h	Lipid peroxides in vitro
Caecum	0	77±6·8	136±11·7	59	76± 8·8	143±13·2	67
	3	76±6·8	128±12·4	52	106±10·8[b]	182±20·4[b]	76
	7	88±7·2	146±13·7	58	134±11·7[b]	252±24·6[b]	118
Blood	0	0·64±0·054	—	—	0·66±0·042	—	—
	3	0·65±0·051	—	—	0·84±0·062[b]	—	—
	7	0·64±0·038		—	1·12±0·078[b]	—	—
Liver	0	186±17·6	406±28·7	220	184±18·5	472±30·6[b]	228
	3	196±16·6	416±28·7	220	268±24·6[b]	612±50·6[b]	394
	7	208±18·9	428±22·4	220	294±16·8[b]	756±42·8[b]	462
Kidney	0	141±12·6	277±22·4	136	156±14·3	296±16·8	140
	3	151±13·7	292±17·6	141	176±9·8	332±21·6	156
	7	162±16·6	286±21·6	124	246±17·5[b]	504±16·8[b]	258

All values are expressed as n mol malonyldialdehyde/g wet weight of tissue or/ml blood and represent the Mean ±S.D. of 6 rats.
[a]Formed after 2 h incubation of homogenates at 37°C.
[b]P value < 0·001 as compared to uninfected.

Table 2. Changes in liver enzymes and their reversal by metronidazole.

Enzymes	Uninfected	Uninfected drug-treated	Infected 3rd day	Infected 7th day	Infected drug-treated
Aminopyrene N-demethylase[a]	1·49±0·099	1·59±0·107	0·949±0·089^g	0·86±0·082	1·42±0·105
Aniline hydroxylase[b]	61·8±5·4	64·2±5·0	44·1±3·3	40·6±3·1	58·6±5·0
Cytochrome P$_{450}$[c]	2·74±0·16	2·62±0·22	1·36±0·11^g	1·02±0·12^g	2·49±0·18
Acid phosphatase[d]	0·115±0·01	0·120±0·012	0·171±0·017^g	0·189±0·015^g	0·135±0·011
Acid ribonuclease[e]	0·021±0·002	0·022±0·0018	0·034±0·003^g	0·035±0·004^g	0·022±0·002
Cathepsin B[f]	0·016±0·0015	0·017±0·002	0·033±0·003^g	0·034±0·005^g	0·017±0·002

[a,b] Assayed in post-mitochondrial supernatant. [c] Assayed in microsomal fraction. [d,e,f] Assayed in total hoomgenate. Enzyme activities are expressed as units/mg protein and represent the Mean ± S.D. from 6 rats. One enzyme unit is defined as (a) n mol of formaldehyde formed/min/mg protein; (b) p mol of p-aminophenol formed/min/mg protein; (c) n mol/mg protein; (d) μ mol of p-nitrophenol released/min; (e) ΔA_{260}/min/mg protein, (f) μ mol of tyrosine released/min/mg protein, and $^g P$ values < 0·01 as compared to uninfected rats.

Table 3. Effect of metronidazole on lipid peroxidation in tissues.

Tissue	Uninfected	Uninfected drug-treated	Infected	Infected + drug-treated
Caecum 0 h	88 ± 7.2	86 ± 8.3	134 ± 11.7^b	76 ± 6.7
2 h	146 ± 13.7	164 ± 14.3	252 ± 24.6^b	146 ± 10.8
Lipid peroxides formed *in vitro*[a]	58	78	118	70
Blood 0 h	0.64 ± 0.038	0.67 ± 0.047	1.12 ± 0.078^b	0.68 ± 0.052
0 h	208 ± 18.9	212 ± 17.4	294 ± 16.8^b	196 ± 13.8
Liver 2 h	428 ± 22.4	438 ± 30.8	756 ± 42.8^b	412 ± 23.4
Lipid peroxides formed *in vitro*	220	226	462	216
Kidney 0 h	162 ± 16.6	168 ± 13.4	236 ± 16.2	148 ± 13.7
2 h	286 ± 21.6	272 ± 17.4	482 ± 18.8^b	258 ± 13.7
Lipid peroxides formed *in vitro*	124	104	246	110

All values are expressed as in table 1 and represent the Mean $\pm$ S.D. from 6 rats.
[a]Formed after 2 h incubation of tissue homogenate at 37°C.
[b]P value < 0.001 as compared to uninfected control.

caecum, liver and kidney homogenate to form lipid peroxides *in vitro* also increases. Since low redox potential is an essential requirement for the survival of trophozoites of *E. histolytica* (Singh *et al.*, 1973) they could survive in an environment of high lipid peroxides in infected caecum due to the presence of or induction of a powerful superoxide dismutase which has been reported to be present in *E. histolytica* (Weinbach *et al.*, 1980). These results suggest the possibility that lipid peroxides formed at the site of infection *i.e.* caecum are released into blood and transported *via* portal circulation to other organs particularly liver, causing secondary damage such as increase in the activity of lysosomal enzymes and decrease in the activity of drug-metabolizing enzyme and contents of cytochrome P_{450}. These biochemical lesions continuing over a prolonged period of caecal infection could make liver susceptible to amoebic infection.

Acknowledgements

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Effect of chronic choline availability on lung and lymph nodes of rat

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Abstract. Male albino rats were given intraperitoneal injections of choline chloride (0·1, 0·33 or 0·5 × lethal dose 50) for a total period of one month and then killed at the end of 30, 90 and 240 days for the study of pathotoxicokinetics of choline. Chronic choline administration in rats caused a decrease in growth rate, a dose dependent modulating effect on the somatic tissue indices of lung and lymph nodes, as well as cellularity of lymph nodes. In another experiment, the effect of choline on mica induced pulmonary lesions was studied. The combined effect of choline and mica caused adenocarcinoma of bronchiolar epithelium and marked lymph-adenopathy with abnormal cells in the lymph nodes at the termination of experiment (330 days). The results of the present investigation suggest that excess choline availability not only produces pulmonary pathological lesions by itself but it also further enhances the lung lesions in altered pulmonary conditions.

Keywords. Choline; mica; lung; lymph nodes; rat.

Introduction

Choline (trimethyl-β-hydroxyethylammonium) is a quaternary ammonium compound widely distributed throughout plant and animal kingdom (Ziesel, 1981). Choline, an essential component of the cell membrane, plays an important role in the maintenance of the integrity of cell architecture. It is also a precursor for the biosynthesis of an important neurotransmitter, acetylcholine (Olson *et al.*, 1958). The role of choline in nutrition has been well recognized particularly in the prevention of fatty liver development in rats (Best and Hershey, 1932; Best *et al.*, 1932). The occurrence of neoplasm has also been observed in liver, lung and other tissues of rats due to prolonged choline deficiency (Copeland and Salmon, 1946; Salmon and Copeland, 1954).

Whereas various studies have concentrated mainly on elucidating the biochemical and pharmacological effects of acute dose(s) of choline, very little information is available on the consequences of chronic choline administration (Cohen and Wurtman, 1975; Wecker *et al.*, 1978; Upreti, 1979; Wecker and Schmidt, 1980). Recent studies have indicated that choline supplementation protects animals from the convulsant and lethal effects of many pharmacological compounds perhaps through a generalized membrane

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Abbreviations used: CC, Choline chloride; LD_{50}, lethal dose 50; ip injection, intraperitoneal injection.

stabilizing effect (Wecker *et al.*, 1982). Many particulate pollutants are known to alter the structure of cell membranes (Kilroe-Smith, 1974). Mica exposure affects the lungs and causes fibrosis in animals and humans (Zaidi, 1969). In our preliminary studies, we have observed that the administration of choline could alter the toxicity of particulate pollutants, possibly acting as a promoter, in the development of pulmonary lesions in rats (Sahu, 1983; Sahu and Shanker, 1984). Being an essential component of cell membrane, the involvement of choline in the genesis of pulmonary lesions caused by particulate pollutants assumes great significance. It is with this intention that the present investigation was attempted to assess the effects of chronic administration of various choline concentrations on pulmonary tissues, as well as its promoting effect, if any, on mica induced pulmonary lesions in rats.

Materials and methods

Young healthy male albino rats, obtained from the animal breeding facility of Industrial Toxicology Research Centre, Lucknow, were used. The animals were fed standard pellet diet (Hind Lever) and water *ad libitum*. Choline chloride (CC) was obtained from Nutritional Biochemical Corporation, Cleveland, USA. The intraperitoneal (ip) lethal dose 50 (LD_{50}) value of CC in male rats was 450 mg/kg body weight with confidence limit ranging from 378–532 mg/kg body weight using log probit analysis (Goodnight, 1979).

The source and particle size of mica dust used in the present experiments were the same as described earlier (Sahu *et al.*, 1978). The chemical composition of mica dust was essentially the same as given elsewhere (Sahu *et al.*, 1984).

Plan of experiment

Evaluation of organ weight: To elucidate the pathotoxicokinetics, 60 animals were divided into 4 groups. The first group of animals received physiological saline and served as control. The other 3 groups received 0·10, 0·33 and 0·50 × LD_{50} (45, 150 and 225 mg/kg body weight) doses of CC, respectively. CC in 1·0 ml sterile distilled water was administered intraperitoneally, 5 days/week to each rat for a total period of one month. The dose of CC was adjusted every week according to the change in body weight. Five animals from each group were killed at the end of 30, 90 and 240 days, respectively, following the initial dosing. Following autopsy lung, peripheral (axial and popliteal) and regional (tracheobronchial, mesentric, portal, lumbar) lymph nodes and other vital organs were removed, weighed individually and relative organ weight calculated. Peripheral and regional lymph nodes were then cut into pieces, minced thoroughly and forced through a sieve into sterile cold medium-199. The total number of nucleated cells were counted and the viability of cells determined using the trypan blue exclusion method.

Experiment II: In the light of the above preliminary observations, a second set of experiment was carried out to study the effect of CC on mica induced pulmonary lesions. The animals were divided into 4 groups. Each animal of the first group received ip injections of CC in 1·0 ml sterile distilled water, 150 mg/kg body weight (0·33

$\times$ LD$_{50}$), 5 days/week for a total period of two months. Each animal of the second and third groups received a single intratracheal injection of 25 mg mica in 1·0 ml sterile 0·15 M sodium chloride solution. Animals of the third group were simultaneously treated with ip injection of CC (150 mg/kg body weight), and the CC administration was further continued for two months as in the first group. The 4th group received 1·0 ml of physiological saline intraperitoneally and served as control. Animals from each group were killed at the end of 90, 180 and 330 days, respectively, following the initial dosing. Following autopsy lung and tracheobronchial lymph nodes were excised carefully and fixed either in 10% formal saline or in Bouin's solution. Paraffin embedded blocks were prepared and sections of 5 μm thickness were prepared. Multiple sections were stained with hematoxylin and eosin, Giemsa stain (Lennert *et al.*, 1978), and by silver impregnation for reticulin.

Statistical methods

Statistical indices were expressed as mean $\pm$ S.E. Comparisons were made with appropriate controls employing students '*t*' test. *P* values less than 0·05 were considered statistically significant.

Results

Absolute and relative organ weights

CC caused a dose dependent decrease in the relative lung weight at 30 days. However, at 90 days the highest dose (0·5 $\times$ LD$_{50}$) produced 91·3% ($P < 0.001$) increase in the absolute weight and relative lung weight (72·3%; $P < 0.01$). No significant change in the lung weight was observed at the end of 240 days.

At 90 days, the absolute weight of peripheral lymph nodes showed an increase of about 55–60% ($P < 0.05$) when administered the two higher doses of CC. The regional lymph nodes showed 16% and 34% ($P < 0.05$) increase in absolute weight at 90 and 240 days, respectively, with 0·5 $\times$ LD$_{50}$ CC dose. When calculated in terms of relative organ weight, both peripheral and regional lymph nodes showed a significant increase (about 35%; $P < 0.05$) at 90 days with the same dose level of CC. The relative weight of regional lymph nodes remained higher even at the end of 240 days.

The lowest dose of CC (0·1 $\times$ LD$_{50}$) did not cause any change in absolute or relative weight of other vital organs of rats. Significant increase in the absolute weight of liver, kidney, and spleen were observed at 90 days with higher doses. However, there was a considerable decline in the relative weight of thymus at 30 days with the 0·5 $\times$ LD$_{50}$ dose and which persisted at the end of 240 days.

Cellularity of lymphoid organs

Peripheral lymph nodes revealed an initial increase of 46, 188 and 224% in the cell counts at 0·1, 0·33 and 0·5 $\times$ LD$_{50}$ dose levels of CC, respectively. However, there was no significant change at later periods with any dose levels. On the contrary, significant decline in the cellularity of regional lymph nodes was encountered at the end of 90 days

with all the 3 doses of CC. At the end of 240 days, the decrease in cell count was 33·9 %
and 38·5 % ($P < 0.05$) with 0·33 and 0·50 × LD_{50} dose levels, respectively. No
significant effect on the viability of lymph node cells at various time intervals were
observed following various doses of CC administration.

Histopathological findings

Choline treatment: Lungs at 90 days showed heavy collections of lymphoid cells
around bronchioles and blood vessels. At places, the alveolar parenchyma revealed
adenomatoid changes along with a few giant cells. These changes were more prominent
at 180 days. Further, at 330 days, the musculature of bronchioles exhibited eosinophilic
characteristic, while the lumen was filled with debris of polymorphonuclear leucocytes
and epithelial cells (figure 1). Upon silver impregnation, the alveolar parenchyma
revealed dark reticulin fibres in abundance (figure 2).

The tracheobronchial lymph nodes at 90 days showed the presence of few small
cavities in the cortico-medullary region. The lesions at 180 days were comprised of
increased mast cell reaction in the paracortical region, while epithelioid cell reaction
was very prominent around the cavities. However, at 330 days the nodes revealed
hyperplastic reaction together with increased mast cell reaction (figure 3). The
medullary sinuses were greatly dilated and filled with foamy cells and mononuclear
cells.

Mica treatment

In general, the lung at 90 days showed the dust cell reaction and larger number of alveoli
were filled with mica laden macrophages. On silver impregnation, quite a few reticulin
fibres were found with in the dust cell reaction areas at 180 days. Later at 330 days, the
pulmonary reaction was in the form of small fibrotic areas situated usually along with
dilated lymphatics. On silver impregnation dust cell granulomata contained reticulin
fibres and collagen fibres. The lymph nodes at 330 days showed hypertrophy together
with a moderate degree of fibrosis in the cortico-medullary region.

Choline and mica treatment

Lung at 90 days showed at places the presence of hypertrophied bronchiolar
epithelium. In addition, the alveoli around bronchioles and blood vessels contained
dark basophilic mononuclear cells along with pigment and mica laden macrophages. At
180 days the adenomatoid changes along with pronounced focal lesions in the
parenchyma were distinct. At 330 days the pink acellular mass along with dust laden
macrophages were found in the alveoli. The bronchiolar epithelium exhibited
characteristic changes of adenocarcinoma together with adenomatoid reaction of
alveolar parenchyma (figure 4). Upon silver impregnation, different type of dark
reticulin fibres and collagen deposition could be seen.

Lymph nodes at 90 days showed the presence of small dust cell reaction, dark
basophilic mononuclear cells and plasma cell in cortico-medullary region. At 180 days
prominent reactions comprising of reticular cells having basophilic cytoplasm along
with small and large lymphocytes were observed in medullary sinuses. Epithelioid cell
granulomata which contained large dark abnormal cells entrapped in reticulin network
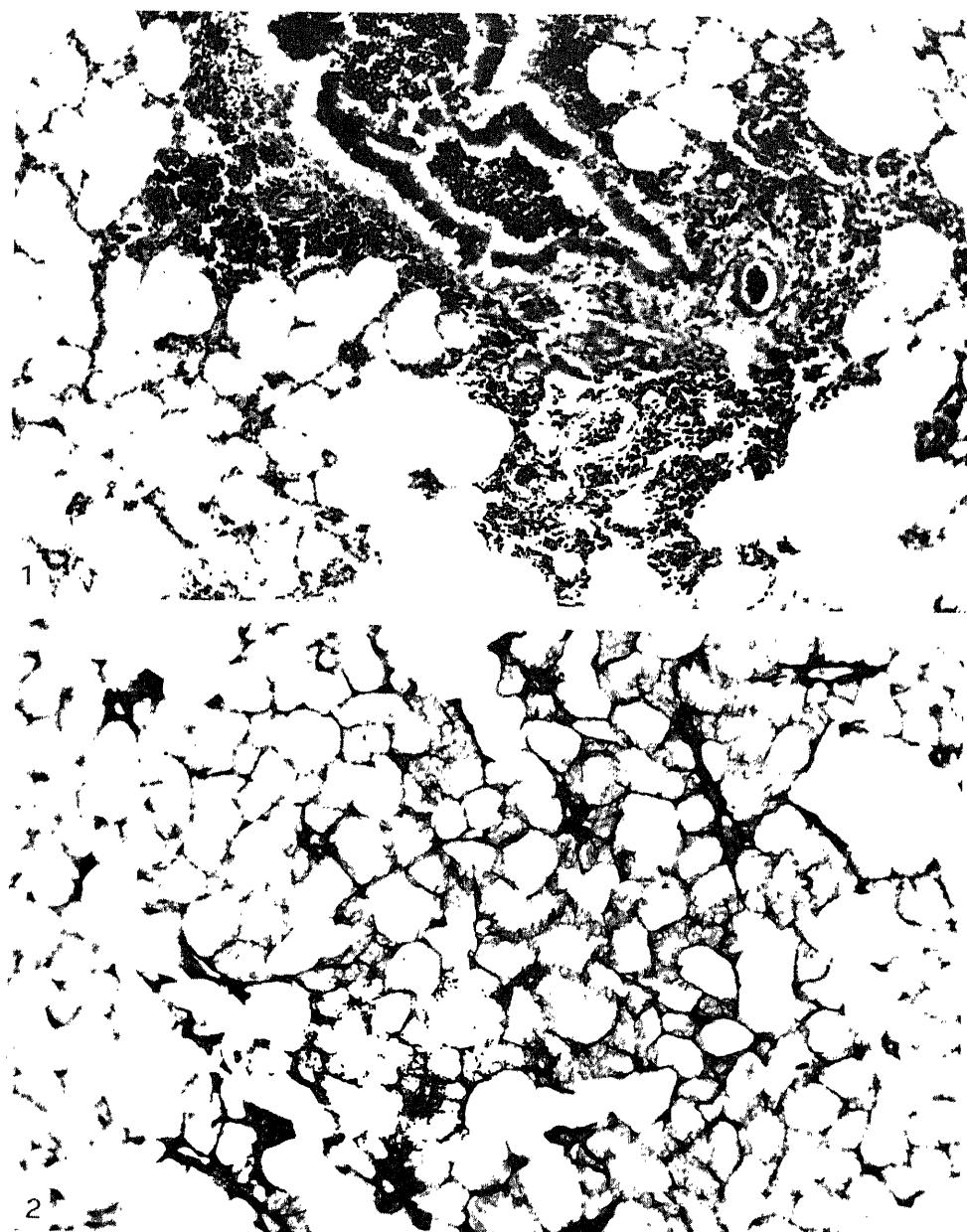
Figures 1 and 2. **1.** Section of lung at 330 days after intraperitoneal administration of choline. Lymphoid cell collection seen around bronchioles together with bronchiolar lumen containing debris comprising of epithelial cells and polymorphonuclear leucocytes. (Hematoxylin and eosin × 256). **2.** Reticulin pattern of choline treated rat lung at 330 days. Dark reticulin fibres are prominent. (Silver impregnation × 256).
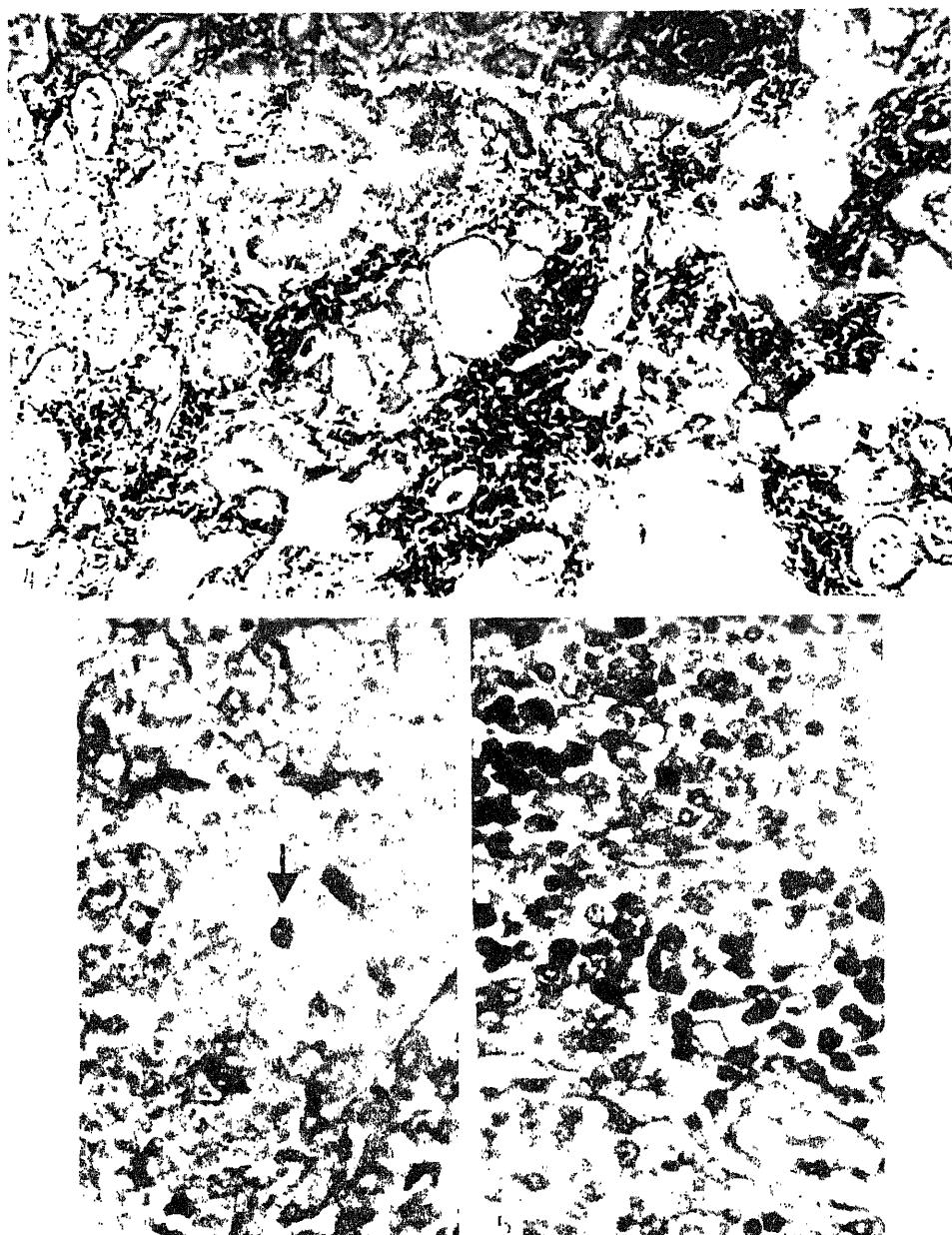
Figures 3–5. **3.** Paracortical region of lymph node of choline treated rat at 330 days
showing hyperplastic reaction with cells fixed in cage like structure and mast cells (arrow).
(Giemsa stain × 485). **4.** Adenocarcinoma and collection of lymphoid cells around bronchi-
oles in the lung of rat at 330 days simultaneously treated with choline and mica. (H and E
× 256). **5.** Masses of large cells having deep blue-gray cytoplasm in the paracortical region of
the lymph node at 330 days in rat simultaneously treated with choline and mica. (Giemsa stain
× 485).

were seen in silver impregnation sections. At 330 days, the lymph nodes were markedly enlarged and possessed thick capsule and also revealed histiocytoid cell reactions as well as blue-grey sheets of large abnormal aggregated cells (figure 5).

No changes of pathological significance were seen in the lung and lymph nodes of control animals upto 330 days.

Discussion

There has been an increasing clinical interest on the likely modulatory effect of the chronic administration of choline or its derivatives for the treatment of neurological and psychiatric disorders. Evidence has also been presented that chronic choline supplementation can prevent the toxicity of numerous pharmacological agents (Wecker *et al.*, 1982). Beneficial as well as adverse pathological effects caused by simultaneous administration of choline are reported in various studies employing different chemicals (Freeman-Narrod, 1977; Ho *et al.*, 1979; Green *et al.*, 1983; Sahu *et al.*, 1984).

The results of pathotoxicokinetic investigations revealed that low as well as high doses of parenterally administered choline to rats generally decreases the growth rate. Further, the study has also brought to light the dose dependent modulating effect of chronic choline administration on the relative weight of lung and lymph nodes. Withdrawl of CC after 30 days revealed insignificant alterations in the relative weights at lower doses. In case of higher doses ($0.5 \times LD_{50}$), the changes in lungs and lymph nodes were significantly higher. This indicates that CC at higher doses may have a prolonged effect. In general, chronic choline administration also affected the cellularity of rat lymph nodes.

In the normal lung there is an extremely rapid turnover of proteins including collagen. This helps in the maintenance of vital function of the lungs in gas exchange and need for rapid adaptation in response to injury. The present histopathological study has demonstrated that on prolonged choline administration, the dilation of lymphatic vessels along with adenomatoid changes of bronchiolar epithelium occurred in the lungs and atypical lymphadenopathy observed as well. There has been considerable evidence to suggest the possible modifying role of choline on respiration as there are cholinergic synapses in the chemosensitive neurons of the ventral surface of the medulla oblongata (Fukuda and Loescheka, 1979). The combined action of mica and choline produced severe fibrotic reaction in the lungs comprising a different type of reticulin and collagen formation and marked lymphadenopathy with abnormal cells in the lymph nodes. Further, the synergistic action caused development of adenocarcinoma of bronchiolar epithelium at 330 days. It is possible that mica dust and choline complement each other in the development of adenocarcinoma of bronchiolar epithelium in lungs at later stages.

It is well known that choline plays an important role in the maintenance of the cell wall integrity. Besides leucine and galactose, choline is also a major precursor of pulmonary surfactant material (Chevalier and Collet, 1972). From our observations it seems probable that chronic choline administration enhances the development of lesions caused by mica in rat lungs and lymph nodes. Further biochemical and electron

microscopic studies at cellular levels are needed to elaborate the mechanism of its dose and time dependent adverse effects in the toxicity of particulate pollutants.

Acknowledgements

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Large scale production and characterisation of dihydrofolate reductase from a methotrexate-resistant human lymphoid cell line

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Abstract. Dihydrofolate reductase has been purified from a methotrexate-resistant human lymphoid cell line (CCRF/CEM-R3) and up to 1 mg of enzyme has been obtained from 5 litres of culture. The enzyme has a molecular weight of 22000 ± 500 as determined by gel filtration. The pH activity profile shows a single optimum at pH 7·7, where marked activation is observed by addition of 0·2 M NaCl. The K_m for NADPH is 3 μM and dihydrofolate 0·7 μM. The binding constant for the inhibitor, methotrexate, is 29 pM.

Keywords. Human dihydrofolate reductase; purification; characterization.

Introduction

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate NADPH oxidoreductase, EC 1.5.1.1.3.) acts in concert with thymidylate synthetase, and both enzymes are required for *de novo* synthesis of thymidylate, a DNA precursor. Inhibitors of these enzymes are among the most useful anti-cancer agents employed clinically. In addition, differences between dihydrofolate reductase from human tissue and micro-organisms have been exploited in the design of bacteriocides and anti-malarials. For the rational design of a new generation of inhibitors, precise details on the mode of inhibition at the atomic structural level are required. The 3-dimensional structures of dihydrofolate reductase from *Lactobacillus casei, Escherichia coli* and chicken lever in a variety of binary and ternary complexes have been reported recently (Matthews *et al.*, 1977, 1978; Volz *et al.*, 1982), but the design of new, clinically useful inhibitors would benefit greatly from a detailed knowledge of the human enzyme. Unfortunately, only very small amounts of dihydrofolate reductase are present in normal human cells and tissues (Jarabak and Bachur, 1971; Lindquist and Bertino, 1976), and none of these tissues would seem a viable source of enzyme for sequence and crystallographic studies.

It is known that cells gradually develop a resistance to the drug Methotrexate (Bertino *et al.*, 1970; Harrap *et al.*, 1971) and one of the mechanisms of the resistance is increased production of dihydrofolate reductase. Alam *et al.* (1983) have produced a methotrexate-resistant human cell line (CCRF/CEM-R3) with over 200-fold enhanced production of dihydrofolate reductase so that sufficient material can be obtained for

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Abbreviations used: SDS, Sodium dodecyl sulphate; M_r, molecular weight.

structural studies. The present work describes the characterisation of dihydrofolate reductase from these cells and its production in milligram quantities.

The CCRF/CEM cell line is a line of lymphoid cells isolated from the peripheral blood of a young girl with acute leukemia (Foley *et al.*, 1965). Since it is conceivable that the dihydrofolate reductase from these cells, or from the methotrexate-resistant overproducing cells, might be different from that obtained from normal cells, it is important that dihydrofolate reductase from other human cell lines should also be characterised and sequenced.

Materials and methods

Materials

Methotrexate, folate, NADPH and other chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, USA; RPMI 1640 medium, donor calf serum and other tissue culture materials from Flow Laboratories Ltd. Aminohexyl-Sepharose 4B from Pharmacia Fine Chemicals and Ultragel AcA44 from LKB-Produkter.

Methotrexate-aminohexyl-Sepharose 4B was prepared by adding 80 mg of solid methotrexate powder to a suspension of AH-Sepharose 4B in distilled water which had been prepared previously by swelling 4 gm of AH-Sepharose 4B in 50 mM NaCl overnight and then washing with distilled water. With the pH adjusted to between 4·5 and 6, solid carbodiimide powder was added to a final concentration of 100 mM. The pH was maintained between 4·5 and 6 for another hour and then the reaction was allowed to proceed for 24 h at room temperature with gentle stirring. The slurry was poured on to a column (2 × 10 cm) and washed with 100 mM $NaHCO_3$ solution overnight at a flow rate of 100–150 ml/h. The column was equilibrated with 50 mM sodium phosphate, pH 6·5.

Cell culture

Cells were grown in suspension in RPMI 1640 medium supplemented with 10 % donor calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml), to a density of 1×10^6 cells/ml. Cells were grown in suspension of 0·5–1 litre. Occasionally larger suspensions were also grown. Cells were harvested by centrifugation at mid-log phase, and the cell pellet was washed with saline buffer pH 7·2, spun down and stored at $-20°C$. A methotrexate resistant human cell line (CCRF/CEM-R3) was produced by treating sensitive, non-resistant cells (CCRF/CEM) with gradually increasing doses of methotrexate (Alam *et al.*, 1983).

Enzyme assay

Dihydrofolate reductase was assayed by spectrophotometric monitoring at 340 nm of the conversion of NADPH to $NADP^+$. One unit of enzyme is defined as that amount of enzyme which oxidizes 1 μmol of NADPH/min in a cuvette of 1·0 cm light path at 30°C, calculated from a molar absorbance change in the reaction of 12350/mol/cm (see, for example, Jarabak and Bachur, 1971). Assays were performed at 30°C in 50 mM Tris/HCl, pH 7·0, containing 200 mM NaCl, 100 μM NADPH and 100 μM dihydro-

folate. For K_i estimation, methotrexate was added to a mixture of enzyme, buffer and NADPH, and the mixture incubated at 30°C for 5 min. The reaction was started by the addition of 50 μM dihydrofolate. Protein was estimated by the method of Lowry *et al.* (1951).

Enzyme purification

Cell pellets were suspended in 20 mM Tris/HCl, pH 7·0, containing 20 mM NaCl and subjected to freezing and thawing 3 times. The lysate was spun at 160,000 *g* for 1 h and the supernatant collected. The cell free extract was brought to pH 5·2 by dropwise addition of 10 M acetic acid with gentle stirring. After 20 min incubation at 5°C the extract was spun at 160,000 *g* for 20 min and the supernatant collected and titrated back to pH 7·0 by dropwise addition of 5 M NaOH. ·A 50–90% ammonium sulphate precipitate was obtained (Green and Hughes, 1955). These acid precipitation and ammonium sulphate fractionation steps each removed about 40% of additional protein.

The 50–90% ammonium sulphate precipitate was dissolved in 1–2 ml of 50 mM sodium phosphate buffer, pH 6·5, and applied to a column of Ultragel AcA44, (figure 1). The active enzyme fractions from gel filtration were applied to a methotrexate-Sepharose column; the enzyme activity was located in a single peak of protein eluted with folate (figure 2). The fraction containing dihydrofolate reductase activity were bulked and concentrated using an Amicon ultrafiltration cell with a
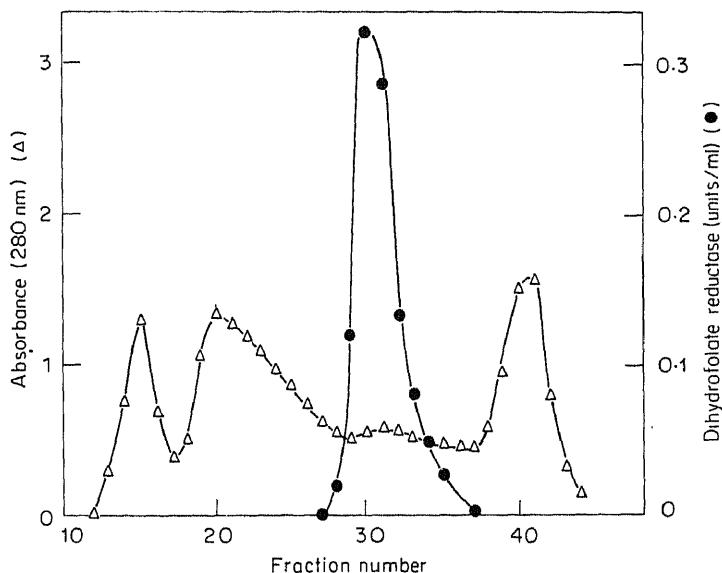
Figure 1. Gel filtration of CCRF/CEM-R3 dihydrofolate reductase on Ultragel AcA44. The column (2·5 × 90 cm) was equilibrated with 50 mM sodium phosphate, pH 6·5. The 50–90% ammonium sulphate precipitate was dissolved in 1–2 ml of buffer and applied to the column. Fractions (11 ml) were collected at a flow rate of 20 ml per h. Fractions were monitored for A_{280} (Δ) and dihydrofolate reductase activity ($\bullet$).
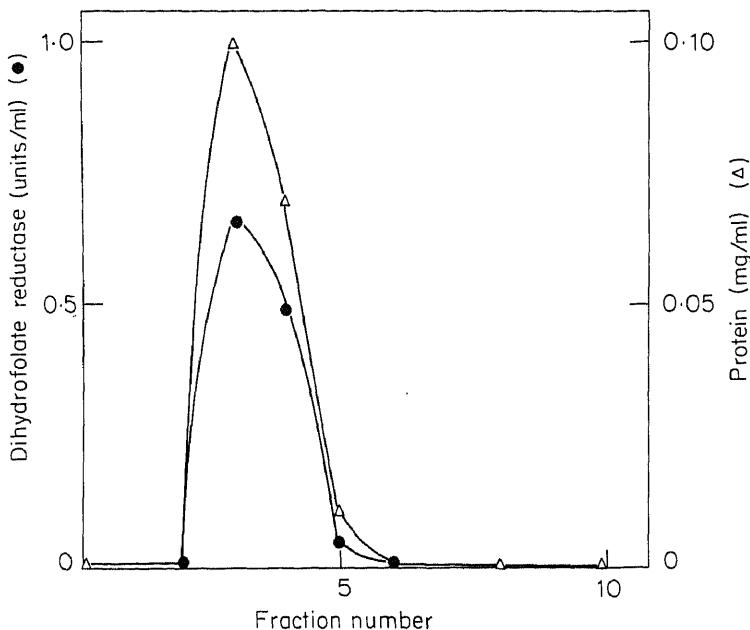
Figure 2. Affinity chromatography of CCRF/CEM-R3 dihydrofolate reductase.

The peak enzyme fractions from the Ultragel AcA44 column were applied to a methotrexate-aminohexyl-Sepharose-48 column at a flow rate of 30 ml/h, followed by 4 bed volumes of equilibration buffer. The column was washed with about 4 bed volume of 50 mM sodium phosphate, pH 6·5, containing 1 M NaCl, at a flow rate of 30 ml/h, and then washed with equilibration buffer until the absorption of effluent at 280 nm was negligible. The enzyme was eluted with 50 mM Tris/HCl, pH 8·5, containing 50 mM NaCl and 1 mM folic acid at a flow rate of 40 ml/h. Fractions were monitored for dihydrofolate reductase activity (●) and protein (Δ).

Diaflo-PM10 membrane. In order to remove folic acid, the concentrated enzyme solution was applied to a Sephadex G-25 column (2·5 × 15 cm) and eluted with 50 mM sodium phosphate buffer, pH 7·2.

Occasionally, it was noticed that some enzyme did not bind to the affinity column and that the binding was not improved by altering the pH or using a fresh affinity column. In these preparations some activity was also lost at the ultrafiltration step. Poor binding of dihydrofolate reductase to affinity column and subsequent loss of activity at the ultrafiltration step resulted in preparations which came from cell cultures grown in quantities in excess of 1 litre suspensions. It was also found that CCRF/CEM-R3 cell line did not grow well (to a density of 1×10^6 cell/ml) in suspensions larger than 1 litre. Therefore, purification yield of dihydrofolate reductase is influenced by the quality of the cell culture. If there is any intracellular damage of the enzyme, this would probably occur before cell lysis since recovery is occassionally low even when the protease inhibitor phenylmethyl sulphonyl fluoride is used during lysis of the cell pellets and the following steps.

Sodium dodecyl sulphate-gel electrophoresis

For gel electrophoresis, a 7–17 % polyacrylamide gel containing 0·1 % sodium dodecyl sulphate (SDS) was used and 50 μg of enzyme was loaded (Laemmli, 1970). Protein was boiled for 5 min in a mixture containing 2 % SDS, 10 % mercaptoethanol, 5 % glycerol and a drop of bromophenol blue solution (figure 3).

Amino acid analysis

The amino acid composition of dihydrofolate reductase was assessed after hydrolysis for 24, 72 and 96 h. Calculations were made using a molecular weight (M_r) of 22,000.

Sequence of N-terminal amino acid

The Beckman Sequencer 890, Beckman Instrument Inc., USA was used for automatic sequencing. The phenylthiohydantoin derivatives of the amino acid were identified using HPLC, Water Ass. ALC/GPC 202, USA and thin-layer chromatography (Rosmus and Deyl, 1972).

Results and discussion

Dihydrofolate reductase, purified as described above, showed a single band of protein on SDS-gel electrophoresis (figure 3). Typically, 1 mg of enzyme was obtained from
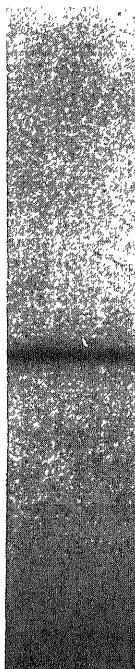
Figure 3. SDS-gel electrophoresis of purified reductase.

5 litres of cell culture. The enzyme may be stored in 0·2 M phosphate buffer, pH 7·0, at − 20°C for several weeks without significant loss of activity.

Estimation of M_r by gel filtration on Ultragel AcA44 using ribonuclease (M_r = 137,000), bovine serum (67,000), ovalbumin (43,000) and chymotrypsin (25,000) as markers, gave a value of 22,000 ± 500. This is similar to the value of 20–22,000 estimated for human placental dihydrofolate reductase (Jarabak and Bachur, 1971) and 21,500 calculated for dihydrofolate reductase from other mammalian species, based on their amino acid sequence (Hitchins and Smith, 1980).

Dihydrofolate reductase from a variety of sources are stimulated by salts (see Hitchins and Smith, 1980). In the present work, sodium chloride gave a maximum (two-fold) stimulation at approx. 200 mM at pH 7·0 (figure 4). The pH optimum for activity was 7·7 (figure 5).

The purified enzyme from the CCRF/CEM-R3 cell line gave a specific activity of 6·5 units/mg, which is about 4 times higher than that reported for human placental enzyme (Jarabak and Bachur, 1971). In the present work only very approximate measurements of specific activity were made prior to affinity chromatography because the inhibitor methotrexate was still present in the preparation. Experience has shown that the enzyme is damaged during the extensive dialysis required to remove methotrexate and this dialysis is not essential since the enzyme exchanges any methotrexate carried over from the cell culture for the methotrexate arms of the affinity column.

The rate of dihydrofolate reduction was measured as a function of concentration of NADPH and dihydrofolate. The K_m value for the two substrates, calculated using the
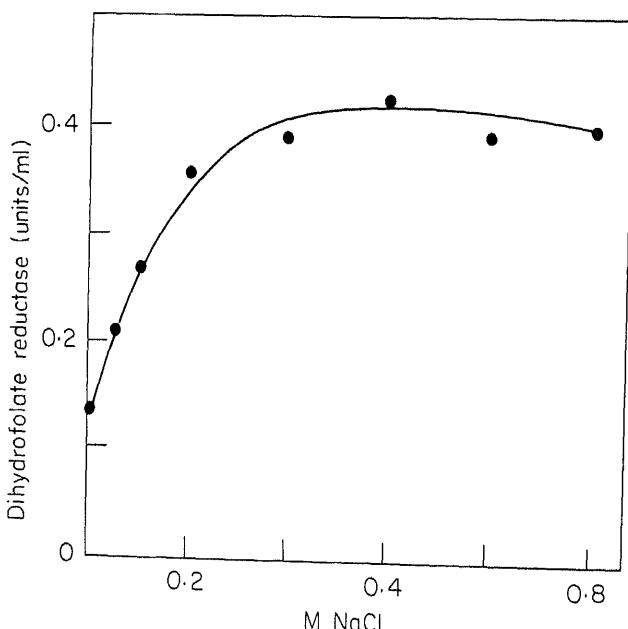
Figure 4. The effect of sodium chloride on dihydrofolate reductase activity.
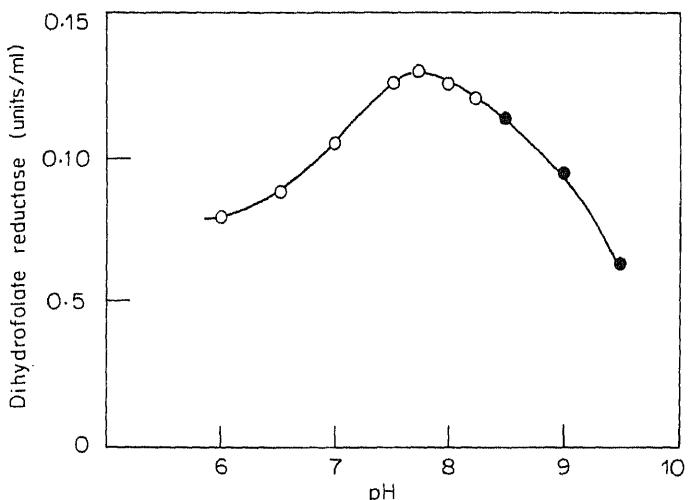
Figure 5. pH-dependence of CCRF/CEM-R3 dihydrofolate reductase.
Buffers used were (O) 200 mM sodium phosphate and (●) 50 mM Tris/HCl containing 200 mM NaCl to maintain uniform ionic strength.

least square method of Marquardt (1963), were 3·0 μM for NADPH and 0·7 μM for dihydrofolate. Inhibition of dihydrofolate reductase was obtained in the presence of 100 μM NADPH and 50 μM dihydrofolate (figure 6). The K_i for methotrexate, calculated using the statistical method of Henderson (1973), was 29 pM. In table 1, these values are compared with those of dihydrofolate from other sources and seen to be closely similar to the values reported for pig liver dihydrofolate reductase (Smith *et al.*, 1979).

The amino acid composition of the purified human dihydrofolate reductase is given in table 2. The presence of 4 methionine residues has been confirmed by cleaving the protein with cynogen bromide; this procedure gives 5 peptides. The value for tryptophane and cysteine were not determined. The M_r calculated from amino acid composition is 21,741 and the value is consistent with the value obtained from the gel filtration.

Recently, the nucleotide sequence of the human dihydrofolate reductase has been determined from the analysis of human dihydrofolate reductase cDNA and the amino acid sequence has been derived (figure 7) (Masters and Attardi, 1983). The N-terminal amino acid sequence determined in this work has been compared with those of corresponding nucleotide and amino acid sequences. The sequence determined is consistent with those derived from nucleotide sequencing studies.

There is only one previously reported study on human dihydrofolate reductase and this shows a wide variation in the values of the kinetic parameters (see table 1). No doubt some of these differences can be attributed to differences in experimental conditions and techniques and it is probably too early to conclude that the enzymes are different. Unfortunately, the cell line studied by Jackson and Neithammer (1977) were later found to be a murine cell line and not a human cell line (Alam *et al.*, 1983). There is

Table 1. Kinetic constants for some of the purified dihydrofolate reductases.

Source	K_m (μM) NADPH	K_m (μM) Dihydrofolate	K_i (pM) Methotrexate	Turnover No. (min^{-1})	Reference
L. casei	0·78	0·36			Dann et al. (1976)
Chicken liver	0·15	1·6			Kaufman and Gardiner (1966)
L12 10 (mouse lymphoma)	1·36	0·3			McCullough et al. (1971)
Beef liver	22	7·5			Rowe and Russel (1973)
Pig liver	3·2	0·74		810	Smith et al. (1979)
Human CCRF/CEM-R3	3·0	0·7	29	963	Present work
Human placenta	2·5	0·11	6	518	Jarabak and Bachur (1971)
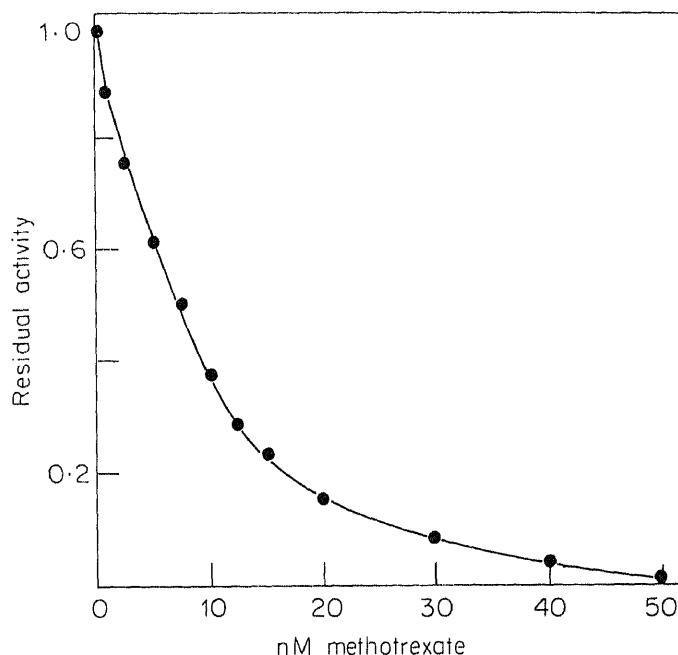
Figure 6. Inhibition of dihydrofolate reductase with methotrexate.

Activity of dihydrofolate reductase was measured in the presence of increasing amounts of methotrexate. Assays were performed as described in the text with 100 μM NADPH and 50 μM dihydrofolate.

Table 2. Amino acid composition of CCRF/CEM-R3 dihydrofolate reductase

Amino acid	Composition (residues/mol)
Asp	23
Thr	12
Ser	15
Glu	27
Pro	6
Gly	14
Ala	13
Cys	—
Val	12
Met	4
Iso	8
Leu	16
Tyr	8
Phe	8
Trp	—
Lys	11
His	9
Arg	7
	190 residues

Top   ATGGTTGGTTCGCTAAACTGCATCGTCGCTGTGTCCCAGAACATGGGCATCGGC
          AAGAACGGGGACCTGCCCTGGCCAC . . . .

Middle   Val Gly Ser Leu Asn Cys Ileu Val Ala Val Ser Gln Asn Met
          Gly Ileu Gly Lys Asn Gly Asp Leu Pro . . . .

Bottom   Val Gly Ser Leu Asn Cys Ileu Val Ala Val Ser Gln Asn Met
          Gly Ileu Gly Lys Asn Gly Asp Leu Pro . . . .

Figure 7.   N-Terminal amino acid sequence of human dihydrofolate reductase.
Nucleotide sequence of the human dihydrofolate reductase cDNA coding region (top) and amino acid sequence of the human dihydrofolate reductase as derived from the nucleotide sequence (middle) (Masters and Attardi, 1983). Amino acid sequence of the CCRF/CEM-R3 dihydrofolate reductase as determined in this study (bottom).

a need for further studies to establish whether the enzyme differs in different human tissues and especially in normal and methotrexate-resistant overproducing cell lines.

Acknowledgements

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Influence of starvation, Triton WR-1339 and [131I]-human serum albumin on rat liver lysosomes

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Abstract. The response of rat liver lysosomes to starvation and administration of lysosomo-tropic agents *viz*. Triton WR-1339 and [131I]-human serum albumin, was assessed in terms of their distribution pattern after isopycnic sucrose density gradient centrifugation. Starvation induced changes in lysosomes appeared to be similar to that produced by the detergent uptake. Both the treatments caused a distinct decline in the equilibration densities of the organelles. On the other hand, injected labelled protein failed to comigrate with the lysosomal markers in starved as well as Triton treated rats and conspicuously remained in a region of high specific gravity in the gradient. These findings indicate retarded fusion between secondary lysosomes and [131I]-human serum albumin containing phagosomes in the livers of rats subjected to starvation or detergent treatment.

Keywords. Lysosome; starvation; Triton WR-1339; [131I]-human serum albumin; isopycnic centrifugation.

Introduction

Susceptibility of lysosomes to dietary restrictions such as starvation is often manifested in terms of specific alterations in size, shape and density of the organelles (Swift and Hruban, 1964; Harikumar and Ninjoor, 1979) which have been attributed to the stimulated autophagic uptake of cellular constituents. Thus, the increase in lysosomal density noted under deprivation induced autophagy in perfused livers of rats has been shown to be due to the sequestration of glycogen and smooth endoplasmic reticulum (Neely *et al.*, 1977). We have demonstrated earlier that the equilibration densities of heterogeneous populations of liver lysosomes in starved rats decrease markedly due to an apparent enhancement in the accumulation of lipid particles in lysosomes (Harikumar and Ninjoor, 1979). In view of the finding that the uptake of Triton WR-1339 by lysosomes promote lipid accumulation (Hayashi *et al.*, 1981) and thereby reduces lysosomal specific gravity (de Duve, 1975; Warburton and Wynn, 1977) an attempt is made in the present work to compare the starvation induced lysosomal changes with that produced by the detergent, to deduce evidence substantiating the above hypothesis. Data on the influence of another lysosomotropic agent, [131I]-

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Abbreviations used: [131I]-HSA, [131I]-Human serum albumin; NAG, N-acetyl-β-glucosaminidase; AS, arylsulphatase; EDTA, ethylene diamine tetraacetic acid; ML, mitochondria lysosome rich; L_1, low density lysosome; L_2, high density lysosome.

labelled human serum albumin ($[^{131}I]$-HSA) on lysosomes are also provided to enable further comparison.

Materials and methods

Para-nitrocatechol sulphate, *p*-nitro-phenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, *p*-nitrophenol and *p*-nitrocatechol were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Analar sucrose and Triton WR-1339 were obtained from British Drug House, Bombay and Ruger Chemical Co., Irvington, New Jersey, USA respectively. $[^{131}I]$-HSA was supplied by the Isotope Division, Bhabha Atomic Research Centre. All other chemicals were of analytical grade quality.

Preparation of denatured labelled protein

$[^{131}I]$-HSA (specific activity 39–46 μCi per mg protein) was denatured (Mego and McQueen, 1967) by treating with 4% formaldehyde solution in 0·05 M sodium carbonate buffer, pH 10·0. After standing in cold (0–4°C) for 3 days, the protein solution was dialysed exhaustively against 0·1 M NaCl. The protein content and the radioactivity were then determined.

Treatment of animals

Male albino rats of Wistar strain weighing 250–300 g, reared on a laboratory stock diet *ad libitum* were used in the present investigation. Animals were starved for indicated time intervals by withdrawing the diet, while they had free access to water throughout the experimental period. Triton WR-1339 was administered intraperitoneally to rats in 0·9% saline at a dose of 85 mg/100 g body weight (Leighton *et al.*, 1968). After administering the detergent, the animals were either fed the stock diet for 5 days or fasted for indicated periods. Formaldehyde denatured $[^{131}I]$-HSA was injected into the tail veins of rats (0·25 mg/100 g body weight). The animals were sacrificed 30 min after the administration of labelled protein. Five rats were included in each of these experiments.

Tissue fractionation

Rats were killed by decapitation. Livers were rapidly excised, washed quickly with the homogenizing medium, blotted and weighed. Liver homogenates (10%, w/v) were prepared in 0·25 M sucrose containing 1 mM EDTA (disodium salt) by two up and down strokes in a Potter-Elvehjem glass homogenizer and filtered through 2 layers of surgical gauge (Harikumar *et al.*, 1978). Fractionation of liver homogenates was carried out as described earlier (Harikumar and Ninjoor, 1979) employing differential and isopycnic sucrose density gradient centrifugation procedures. A mitochondrial-lysosome rich (ML) fraction isolated from liver homogenate by differential centrifugation was resuspended in 10% (w/v) sucrose and layered on the top of a continuous gradient (42 ml) of 80% (w/v) and 35% (w/v) sucrose solutions. The samples were spun at 25,000 rev/min in a Beckman-Spinco Model L2-65 B ultracentrifuge using SW 25-2 rotor for 90 min and the fractions (2 ml) were collected from the top of the gradient by aspiration with a glass syringe.

Analytical procedures

Distribution of radioactivity in tissue preparations was measured employing a Gamma Counter (Medical Spectrometer, Type NI-2123, BARC). Lysosomal marker enzymes, arylsulphatase (AS) [EC 3.1.6.1] and N-acetyl-β-D-glucosaminidase (NAG) [EC 3.2.1.30] were assayed as described by Barrett (1972) after subjecting tissue samples to 8 freeze thaw cycles. Protein was measured according to the method of Lowry *et al.* (1951).

Results and discussion

In the present investigation the characteristic properties of Triton WR-1339 and [^{131}I]- HSA in decreasing (Warburton and Wynn, 1977) and increasing (Davies, 1973) lysosomal densities respectively, are taken advantage of in eliciting information regarding the starvation induced alterations in rat liver lysosomes. As shown in figure 1, fasting for 5 days as well as administration of the detergent led to a pronounced shift in the equilibration densities of lysosomal marker enzymes NAG (left panel) and AS (right panel). Starvation resulted in a distinct bimodal distribution of NAG with nearly 85% of the enzyme activity concentrated in two regions of the gradient *i.e.* fractions 9–13 ($\rho = 1 \cdot 059 – 1 \cdot 146$) and fractions 15–21 ($\rho = 1 \cdot 166 – 1 \cdot 230$). These two regions apparently
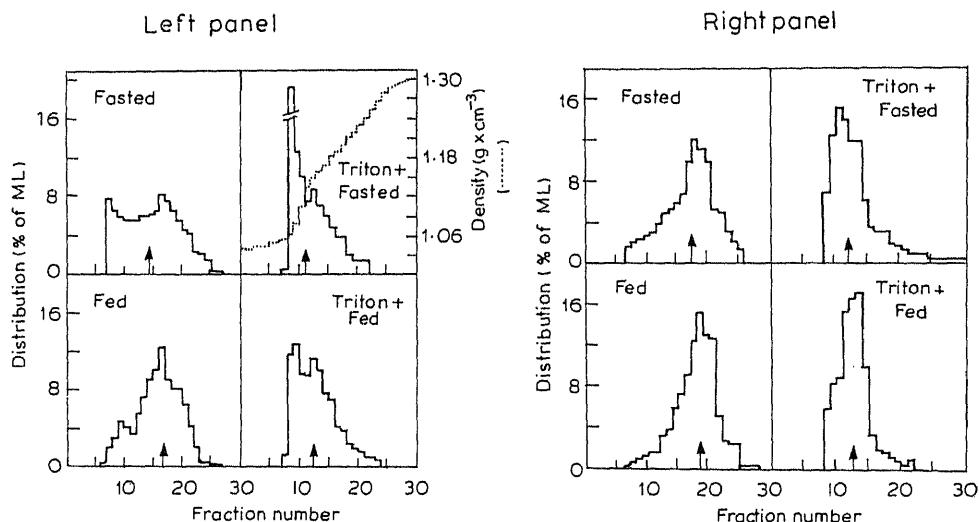
Figure 1. Influence of starvation (5 days) and Triton WR-1339 on the distribution profiles of NAG (left panel) and AS (right panel) after isopycnic density gradient centrifugation. While fractions 1–11 exhibited a non-linear density distribution between $1 \cdot 038$ and $1 \cdot 04$ g × cm^{-3}, fractions 12–30 showed a linear distribution in the range of $1 \cdot 132 – 1 \cdot 298$ g × cm^{-3}. The histograms show averages of results obtained from 3 independent experiments. The arrows indicate the position of median densities.

Table 1. Yield of marker enzymes in lysosomal populations isolated by isopycnic density gradient centrifugation.

| | Yield of enzymes (%) | | | |
| | AS | | NAG | |
Treatments	L_1	L_2	L_1	L_2
Control	9·2	57·1	12·4	72·6
Fasted, 5 days	14·0	63·6	30·6	57·2
Triton + fed, 5 days	55·4	19·7	56·0	30·0
Triton + fasted, 5 days	60·7	22·0	64·6	25·1

Yield of marker enzymes is expressed as % of the enzyme activity in ML fraction. L_1 represents populations equilibrated in fractions 9–13 ($\rho = 1·059–1·146 \text{ g} \times \text{cm}^{-3}$). L_2 represents populations equilibrated in fractions 15–21 ($\rho = 1·166–1·230 \text{ g} \times \text{cm}^{-3}$).

representing dual populations of lysosomes, were termed as low density lysosomes (L_1) and high density lysosomes (L_2) respectively. Density alterations in lysosomes induced by starvation and Triton WR-1339 were evaluated in terms of the relative yield of marker enzymes in these two populations. Unlike NAG, AS did not exhibit bimodal distribution. However, in view of the marked shift in median density of the enzyme from high density region to lower densities (figure 1), the distribution of this enzyme was also assessed in L_1 and L_2. The density lowering effect of the detergent (Warburton and Wynn, 1977; de Duve, 1975) is obvious from the decline noted in the median densities (1·204 to 1·142 $\text{g} \times \text{cm}^{-3}$ for AS and 1·195 to 1·139 $\text{g} \times \text{cm}^{-3}$ for NAG) of the lysosomal populations (figure 1) concomitant with the perceptible increase in the yield of L_1 (table 1). It is interesting to note that despite differences in the degree of increase in the yield of L_1 during starvation (4–18 %) and Triton treatment (45 %), a translocation of lysosomal populations to markedly reduced density regions (figure 1) is a characteristic feature of both starvation and detergent loading. It may therefore be pointed out that Triton loading simulates the effects of starvation at least with respect to causing changes in lysosomal densities. These results assume significance in the light of available information on the involvement of lipids even in the presence of other cellular materials like proteins (Gregoriadis and Ryman, 1972) in lowering the lysosomal density, and their relatively longer retention in lysosomes (Glaumann et al., 1979) and lead us to conclude that both the treatments promote accelerated accumulation of lipid particles in the organelles. When Triton administration was coupled with 5 days starvation, a further narrowing of the distribution histograms (figure 1) and an enhancement in the yield of L_1 (table 1) was observed suggesting that the effects of fasting and detergent are inter-related and additive in nature. This explanation is valid in view of the reported observations that Triton filled lysosomes fuse with preformed phagolysosomes rather than with newly synthesized lysosomes (Tsung et al., 1975). While the property of Triton WR-1339 in triggering rapid inclusion of lipids in lysosomes is undisputed, the origin of lipids and the significance of their

enhanced accumulation in lysosomes during starvation remain obscure. It is possible that the extra energy demand of the cell in the absence of dietary supply is met by the mobilization of lipids from the fatty resources (Nepolitano, 1963) and/or by the direct utilization of cellular constituents (Levy and Elliot, 1968) subsequent to their sequestration into lysosomes. Since lipid degradation in lysosomes is comparatively a slower process (Glaumann *et al.*, 1979) both the above events could lead to a progressive accumulation of undigested lipids affecting the buoyant densities of the organelles. We have observed (Brij Bhushan *et al.*, 1981) that fasting enhances lipid storage in rat liver, mainly in the form of triglycerides. How much of this increase could be attributed to inclusion inside lysosomes is however, not known.

Unlike Triton WR-1339, the endocytosis of $[^{131}I]$-HSA seems to exert hardly any influence on the equilibration densities of lysosomes isolated either from fasted or fed livers (figure 2). In both the groups, the labelled protein appears as a single sharp peak at the high density region ($1 \cdot 166$–$1 \cdot 230$ g $\times$ cm^{-3}). The starvation effect is discernible to the extent that there is a 13 % increase in the yield of radioactivity accompanied by an elevation in the peak height of the label. It is however, noteworthy that the marker enzymes tend to migrate towards lower densities, away from the protein peak as a consequence of starvation indicating an inadequate fusion of protein containing phagosomes with the existing lysosomes. This was further substantiated by the data incorporated in figure 3 and table 2 where the lysosomal response elicited by both Triton WR-1339 and $[^{131}I]$-labelled protein was ascertained in livers of rats fed or fasted for varying periods. It was observed that the particulates containing labelled
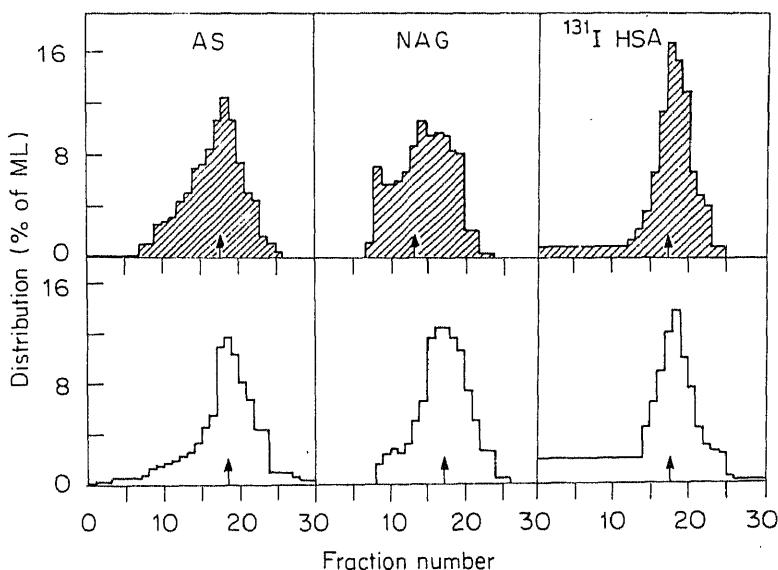

Figure 2. Equilibration profiles of $[^{131}I]$-HSA and liver lysosomal marker enzymes during isopycnic density gradient centrifugation in fed, control □ and fasted (5 days) ▨ rats. The distribution of $[^{131}I]$-HSA represents total radioactivity and is expressed on the basis of radioactivity in ML fraction which is considered as 100%.
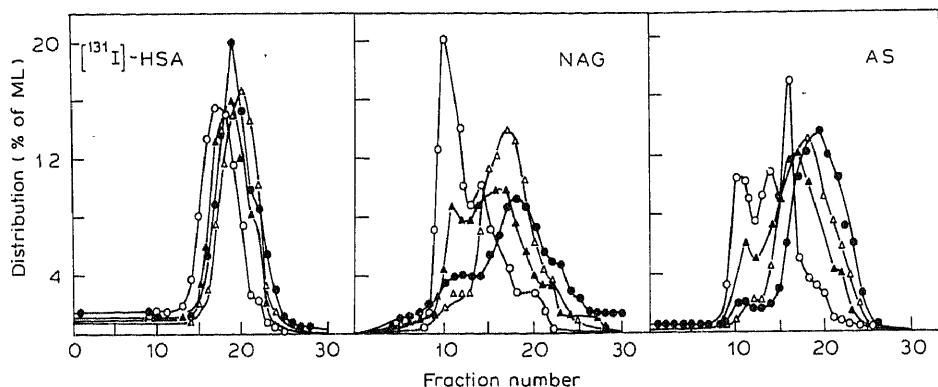
Figure 3. Alterations in the distribution profiles of lysosomal marker enzymes in response to starvation for varying periods in rat livers treated with Triton WR-1339 and [^{131}I]-HSA. Rats were administered Triton WR-1339 and fasted for 1 day (Δ); 2 days (▲) and 5 days (O). These animals along with those belonging to fed control (●) group have been administered [^{131}I]-HSA exactly 30 min prior to sacrifice. The distribution of [^{131}I]-HSA represents total radioactivity and is expressed on the basis of radioactivity in ML fraction which is considered as 100%.

Table 2. Influence of starvation and lysosomotropic agents on the median equilibration densities of lysosomes.

Treatment	Median density (g × cm^{-3}) [^{131}I]-HSA	AS	NAG
Control	1·195	1·201	1·189
Triton + fasted, 1 day	1·204	1·190	1·180
Triton + fasted, 2 days	1·196	1·177	1·167
Triton + fasted, 5 days	1·181	1·153	1·120

The median equilibration densities of the lysosomal marker enzymes and [^{131}I]-HSA were computed from the results presented in figure 3.

protein consistently remain as a single peak in the region of higher specific gravity away from lysosomal marker enzymes in response to starvation and Triton treatment. Administration of labelled protein does not seem to influence the density lowering effects of fasting and detergent treatment. This is apparent from the progressive shift in median densities of NAG and AS to lower values as the period of starvation advanced from 1–5 days (figure 3 and table 2). It is evident from these results that autophagosomes formed in response to starvation fuse readily with tritosomes but not with phagosomes containing [^{131}I]-HSA. These findings taken together with our observation on the diminished intracellular degradation of labelled protein in livers of starved rats (Harikumar and Ninjoor, 1985) are in conformity with the reports on the selectivity associated with the fusion process between secondary lysosomes and vesicles containing externally administered macromolecules (Tsung *et al.*, 1975) and cellular structures

(Glaumann and Trump, 1975). No obvious explanation for the nonfusion of phagosomes containing [^{131}I]-labelled protein with the existing phagolysosomes is available at present. Nevertheless, it is conceivable that the accentuated accumulation of lipids in tritosomes and fasted lysosomes could be responsible for the delayed transfer of labelled protein to secondary lysosomes, possibly due to charge variations in the lysosomal microenvironment. Recently, Hostetler *et al.* (1985) have demonstrated that in chloroquine induced fatty liver, the surface charges of the lysosomes are markedly altered as a consequence of lipid inclusion. Warburton and Wynn (1977) have in fact reported that newly endocytosed material preferentially enters those lysosomes which contain low levels of lipids. Also the functional specificity of the heterogeneous populations of lysosomes (Harikumar and Ninjoor, 1979; Pertoft *et al.*, 1978) as well as disturbances in the cytoplasmic endowments such as microtubules which regulate the intracellular translocation and fusion of vesicles (Dunn *et al.*, 1980; Collot *et al.*, 1984) could influence the phagolysosome formation.

Acknowledgement

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γ-Protein, a sulphur amino acid rich protein from pigeon pea (*Cajanus cajan* (L.) Millsp.)

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Abstract. A globulin protein comparatively rich in sulphur amino acids has been isolated from the seeds of pigeon pea. This protein termed γ-protein has a sedimentation coefficient of 7S and a molecular weight of about 90,000. Antibodies were raised against pure γ-protein. Using rocket immunoelectrophoresis it was observed that γ-protein was synthesised in the developing seeds, 21 days after flowering.

Keywords. Pigeon pea; seed globulin; γ-protein, sulphur amino acids.

Introduction

Grain legume seeds are next to cereals in importance as human food and animal feed. Though they contain a higher amount of protein than the cereals, sulphur amino acids are usually limiting in legumes. One of the approaches suggested for the improvement of seed protein quality in grain legumes is to identify proteins that are relatively rich in sulphur amino acids and to increase their amount by breeding. γ-Conglutin was reported to be one such protein in *Lupinus* (Blagrove and Gillespie, 1975). This suggested us to look for similar proteins in other legumes. In an earlier communication, we had reported the presence of a sulphur rich protein termed γ-protein from pigeon pea (Gopala Krishna *et al.*, 1977). In this paper we report the isolation, characterisation and synthesis of γ-protein from pigeon pea seeds.

Materials and methods

Preparation of seed meal

Seed meal from mature dry seeds (cv. T-21) was prepared by grinding in a Udy cyclone mill fitted with a 40 mesh sieve. The finely milled meal was defatted with cold petroleum ether, air dried and stored at 4°C.

Protein extraction from seed meal

Defatted meal was extracted with 0·01 M sodium borate buffer, pH 8·2 in cold for 3 h with constant agitation. The ratio of seed meal to buffer used was 1:10 (w/v). The slurry

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Abbreviations used: CAM, Cellulose acetate membrane; DAF, day after flowering; SDS, sodium dodecyl sulphate; M_r, molecular weight.

was passed through 4 layers of cheese cloth before centrifugation at 10,000 *g* for 30 min. The supernatant referred to as alkaline extract was used for globulin preparation.

Preparation of globulin

The alkaline extract was diluted with water to make the buffer concentration 0·05 M or lower. The pH of the solution was brought down to 4·8 with slow addition of 6 N HCl and the solution was continuously stirred. The precipitated globulin protein was collected by centrifugation at 12,000 *g* for 15 min. The protein pellet was dissolved in 0·05 M Tris-HCl buffer, pH 8 containing 0·5 M NaCl. The protein solution was loaded onto a Sepharose 6B column (2·8 × 92 cm) equilibrated with Tris buffer mentioned above. The column was eluted at a flow rate of 24 ml/h and 12 ml fractions were collected using an ISCO fraction collector. The column eluant was continuously monitored at 280 nm using a LKB Uvicord II. The void volume fraction was rejected and rest of the 280 nm absorbing fractions were pooled. Globulin protein was precipitated from the pooled fraction by overnight dialysis against 25 mM citrate buffer, pH 4·7. The protein pellet after centrifugation was dissolved in 0·15 M phosphate buffer, pH 7·2 and stored at 0°C.

γ-Protein purification from globulin

The procedure followed for purification of γ-protein from globulin is shown in figure 1.

Estimation of protein

Protein was estimated either by the method of Lowry *et al.* (1951) or by the Biuret method (Layne, 1957). A standard graph was prepared using bovine serum albumin (fraction V) as the standard protein.

Cellulose acetate membrane electrophoresis

Electrophoresis on cellulose acetate membrane (CAM) was carried out in a Beckman microzone electrophoretic apparatus essentially according to the method of Blagrove and Gillespie (1975).

Immunological methods

Antibodies against γ-protein were raised in rabbits (Belgium strain; 4 month old) essentially according to the method described by Jurd (1982). The animals were bled according to the method of Gordon (1981). The serum was directly used without any purification.

Double immunodiffusion was carried out at 4°C for 16–24 h in 1 % (w/v) agarose gel following the method described in the LKB application note 249.

Rocket immunoelectrophoresis was performed in a LKB-Multiphor apparatus according to the method described in the LKB application note number 249. The agarose gel contained 0·2 % (v/v) antiserum. The seeds, (50 for 7 days after flowering (DAF), 20 for 14 DAF and 5 seeds each for 21, 28, 35 and 42 DAF) were extracted in 2 ml of 0·1 M borate buffer, pH 8·2. The homogenate was centrifuged at 12,000 *g* for

Globulin
|
DEAE-Sephacel chromatography
|

Unbound fraction   Bound fraction
|
40% $(NH_4)_2SO_4$
saturation
|
Overnight dialysis in cold
|
Centrifugation
|

Supernatant   Precipitate
|
Dissolved in 50 mM Tris-HCl, pH 8
containing 0·5 M NaCl
|
Sephacryl S-300 chromatography
(1·4 × 60 cm) above buffer
|
Appropriate fractions pooled after
checking purity by CAM
|
Concentrate by precipitation
|
Pure γ-protein

Figure 1. Outline of procedures used for preparation of γ-protein from globulin of pigeon pea seeds.

10 min. The supernatant was used for electrophoresis. For 7 and 14 DAF samples 10 μl of the supernatant and for all later samples 5 μl of the supernatant was used.

Sucrose density gradient centrifugation

Linear sucrose density gradients (10–30% (w/v) in 4·8 ml in volume) were prepared according to the method of Stone (1974). 2 mg protein sample (0·2 ml) in 0·05 M phosphate buffer, pH 7·2 containing 0·4 M NaCl was layered at the top. Centrifugation was for 16 h at 114,000 g and 4°C in a Beckman L5 65B ultracentrifuge. Sedimentation coefficients were determined as described by Martin and Ames (1961).

Column chromatography

A column of Sepharose 6B ($1·6 \times 56$ cm) in 0·05 M Tris-HCl buffer, pH 8 containing 0·4 M NaCl was used for molecular weight determination by gel filtration. The column was eluted at a flow rate of 12 ml/h and 1·2 ml fractions were collected. Effluent fractions were monitored at 280 nm in a Beckman DB-G spectrophotometer. The column was calibrated by determining the elution volume (V_e) of standard proteins and a calibration graph was obtained by plotting V_e against the log molecular weight of the protein.

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of 0·1 % sodium dodecyl sulphate (SDS) was carried out in 10% separating gel slabs ($16 \times 12 \times 0·2$ cm) following the method of Weber and Osborn (1969).

Amino acid analysis

The method of protein hydrolysis and analysis was as described earlier (Gopala Krishna *et al.*, 1977).

Results

When globulin protein fraction in 15 mM phosphate buffer, pH 8 was loaded on a DEAE-Sephacel column equilibrated with the same buffer, the γ-protein did not bind to the ion-exchange resin. On adding ammonium sulphate to 40% saturation the γ-protein precipitated. At this stage the γ-protein still had some contamination which were seen as fast moving zones on CAM. The contaminating protein could be removed
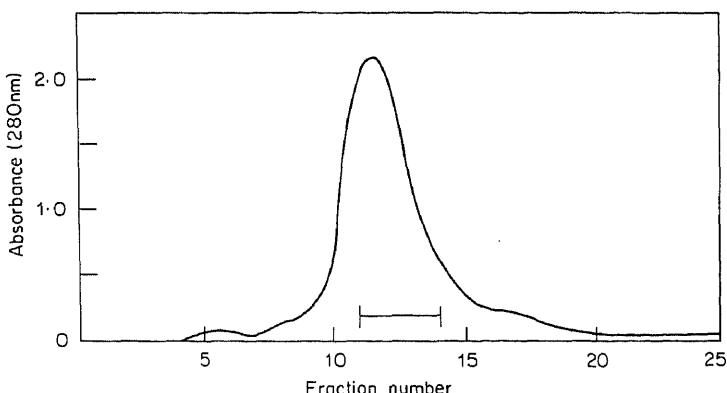
**Figure 2.** Elution profile of γ-protein from a Sephacryl S-300 column ($1·4 \times 60$ cm). Column chromatography was performed in 0·05 M Tris buffer, pH 8 containing 0·5 M NaCl. The column was eluted at a flow rate of 11 ml/h and 4·4 ml fractions were collected. Absorbance at 280 nm was monitored in the different fractions. The horizontal bar indicates the fractions pooled for further analysis.

by passing through a Sephacryl S-300 column. The elution pattern of the protein is shown in figure 2. There was a small protein peak in the void volume and a major peak corresponding to the γ-protein fraction. The purity of each fraction in the peak region was checked by CAM electrophoresis.

The elctropherogram of globulin and the purified γ-protein after CAM electrophoresis is shown in figure 3. The purified γ-protein showed a single band and was free of the fast moving protein contaminants. The protein purity was also checked by gel electrophoresis in a continuous system. The γ-protein although gives one zone, does not travel very much at pH 7·2 because of the low charge to mass ratio (data not shown). Further evidence for the purity comes from the immunodiffusion studies where antibodies raised against purified γ-protein failed to crossreact with either purified legumin or vicilin (figure 4).

Precipitin patterns obtained after rocket immunoelectrophoresis of the protein extracts from seeds harvested at different periods after flowering are shown (figure 5).
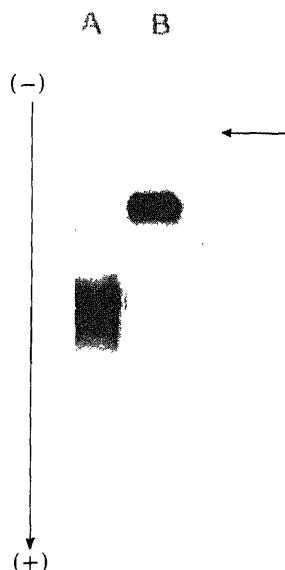

Figure 3. Electrophoresis on CAM of globulin and γ-protein from pigeon pea seed meal. Arrow indicates the origin. Migration was from cathode towards anode. The lanes are (A) globulin (B) γ-protein.
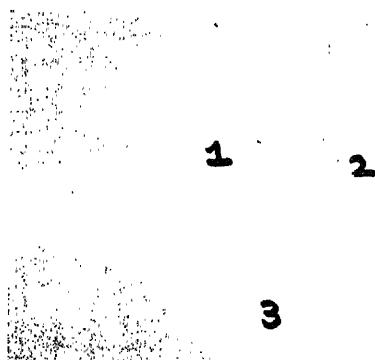
Figure 4. Specificity of antibodies raised against purified γ-protein. Double diffusion was performed in 1% agarose gel for 24 h. The central well contained the antibodies. The peripheral wells contained legumin (1), vicilin (2) and γ-protein (3).
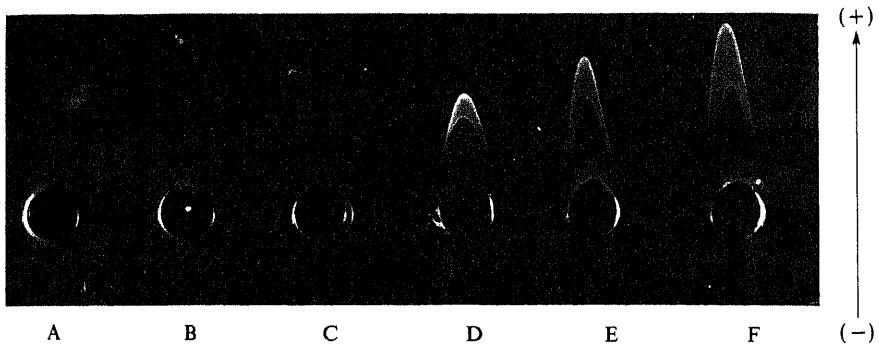
Figure 5. γ-Protein synthesis during different stages of seed development. Rocket immunoelectrophoresis was performed in 1% agarose gel containing 0·2% antisera (v/v). Electrophoresis was performed for 16–18 h at a constant voltage of 200 V. 7, 14, 21, 28, 35 and 42 day old seeds were extracted and were added to the wells (*a–f* respectively).

No precipitin band was observed upto about 3 weeks after flowering. The γ-protein was laid down in the seeds from 4th week onwards and the amount increased till seed maturity.

The molecular weight of the holoprotein was estimated by gel filtration chromatography and by sucrose density gradient centrifugation. On a Sepharose 6B column the γ-protein eluted as a single peak (figure 6) and the molecular weight was estimated to be about 90,000. The γ-protein on sucrose density gradient showed a peak (figure 7) with a sedimentation coefficient of 7S indicating a molecular weight (M_r) of about 130,000.

On SDS-polyacrylamide gels the γ-protein under dissociated but non reduced conditions gave a single band of M_r of about 52,000 while on reduction with 2-mercaptoethanol showed two subunits of molecular weight of about 32,000 and 20,000 (figure 8). Thus the interaction between the two subunits is through disulphide linkages.
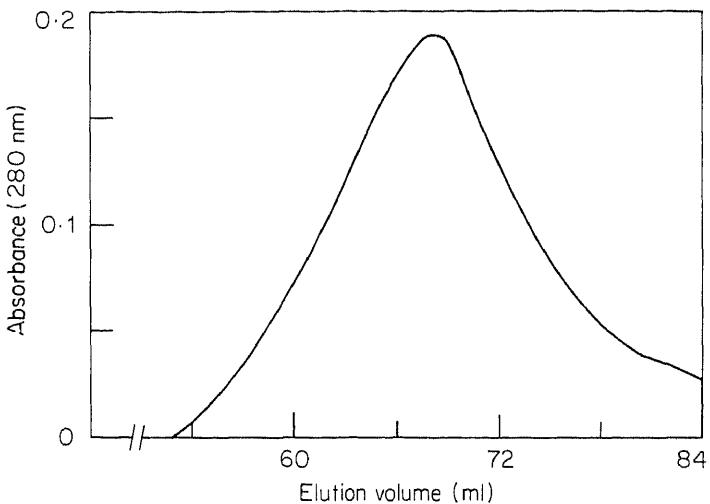
Figure 6. Elution profile of purified γ-protein from a Sepharose 6B column (1·6 × 56 cm). The column was eluted at a flow rate of 12 ml/h and 1·2 ml fractions were collected. Other details are as in figure 1.
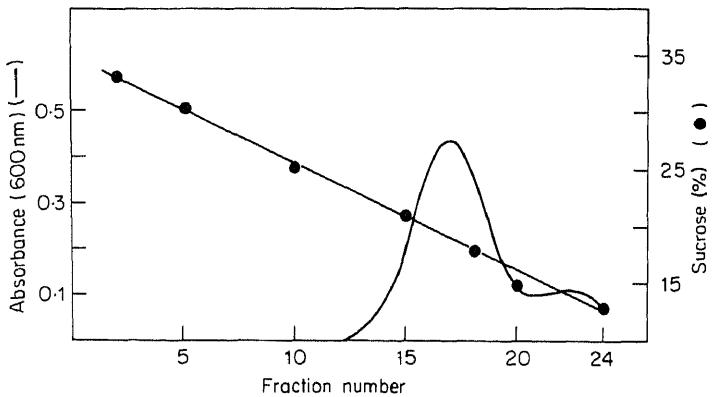
Figure 7. Separation of the purified γ-protein from pigeon pea seed meal according to its sedimentation velocity on sucrose gradients. Gradients were 10–30% sucrose in 0·05 M phosphate buffer, pH 7·2 containing 0·4 M NaCl. Centrifugation was performed at 4°C for 16 h at 40,000 rpm in a Beckman ultracentrifuge. Protein in the fractions was estimated at 600 nm. (●), Indicates the sucrose gradient.

The sulphur containing amino acid composition of globulin and the purified fractions is shown in table 1. The γ-protein has about 4 and 3 times more total sulphur containing amino acids than legumin and vicilin respectively. Thus of the 3 globulin fractions, the γ-protein has the highest amount of total sulphur amino acids.

$$MW \times 10^{-3}$$

94

67

43

30

20.1

14.4

(A) (B)
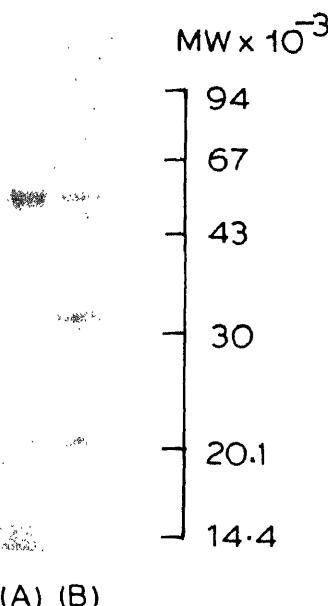
Figure 8. SDS-polyacrylamide gel (10%) electrophoresis of γ-protein in the absence of (A) and presence (B) of 2-mercaptoethanol. About 20 μg protein was loaded in each track after treatment with SDS. The numbers at the side indicate the position occupied by marker proteins.

Table 1. Amino acid composition of globulin and the γ-fraction (g/16 g N).

Amino acid	Globulin	γ-Protein
Lysine	4·40	3·17
Histidine	2·76	1·33
Ammonia	6·13	7·57
Arginine	4·40	2·59
Methionine sulfoxide	0·13	0·22
Aspartic acid	6·12	4·67
Threonine	2·67	2·26
Serine	3·43	2·85
Glutamic acid	11·15	5·03
Proline	3·32	2·70
Glycine	2·52	2·24
Alanine	2·99	2·28
Half Cystine	0·45	1·72
Valine	2·93	3·25
Methionine	0·81	0·85
Isoleucine	2·57	2·14
Leucine	5·27	3·65
Tyrosine	2·37	2·21
Phenylalanine	4·91	2·50
Methionine sulfone	0·18	0·33

Discussion

Pigeon pea is an important grain legume crop in India, parts of Africa and Latin America. We have purified and characterised a globulin protein in pigeon pea seeds which is termed γ-protein. This protein resembles the γ-conglutin from *Lupinus* (Blagrove and Gillespie, 1975). The molecular weight of γ-protein as estimated by gel filtration was 90,000 and this value is higher than the value obtained by adding the molecular weights obtained for the subunits by dodecyl sulphate gel electrophoresis. The γ-protein probably is present as a tetramer (M_r 100,000) consisting of two dimers (M_r 52,000) each which is made up of two subunits of molecular weights 31,900 and 20,000. Blagrove and Gillespie (1975) found that in aqueous solutions conglutin-γ, a protein resembling γ-protein, was made up of a number of polymeric species in contrast to other globulins. They could not find appropriate conditions of salt concentrations and pH to make conglutin-γ assume one polymeric size. A similar situation probably exists for the γ-protein of pigeon pea. However, under the experimental conditions employed for gel filtration, the γ-protein could be existing as a tetramer. This would account for the differences in the molecular weights observed in the different techniques.

Antibodies raised against the γ-protein has been used to develop a simple procedure to investigate the variation in the amount of γ-protein in the germplasm collection. So, now it is possible to use this technique in a breeding programme for selecting lines with increased amounts of γ-protein in pigeon pea.

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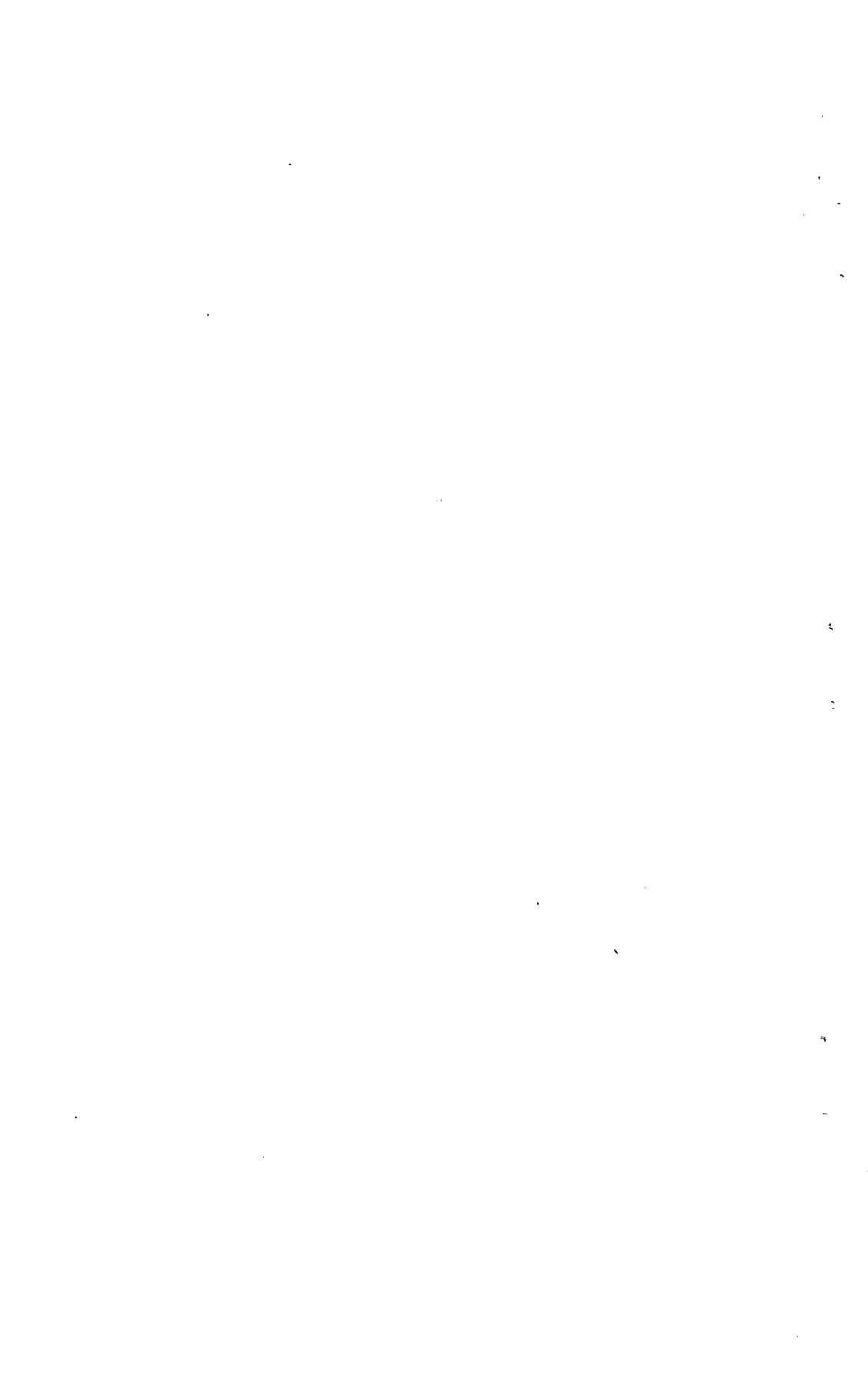
℮ effect of luteinizing hormone releasing hormone and
i-luteinizing hormone releasing hormone antibodies on chorionic
ιadotropin and progesterone secretion by human placental villi
ιitro

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Abstract. Gonadotropin releasing hormone has been located and found to be secreted by the human placenta in culture. Addition of the releasing hormone upto 1 μg concentration in the placental cultures brings about stimulation of chorionic gonadotropin and progesterone secretion. Higher amounts of the decapeptide has an inhibitory influence on both the gonadotropin and the steroid production. The action of the releasing hormone on the placenta could be blocked by the anti-luteinizing hormone releasing hormone monoclonal antibodies indicating a possible site of action of the antibodies for control of fertility.

Keywords. Luteinizing hormone releasing hormone; culture; human chorionic gonadotropin; progesterone; anti-LHRH antibodies.

ιduction

ɔrimate placenta in addition to serving the function of transport of metabolites for eveloping embryo, also serve as an endocrine gland by elaborating the peptide and idal hormones. Thus, functionally it seems to be a composite of hypothalamus, tary and ovary, secreting the hormones produced by each of these tissues. Though tion of pituitary gonadotropins is known to be dependent on releasing hormone, rs controlling the placental hormone synthesis and secretion are poorly rstood.

recent years considerable information has accrued on the presence of a dotropin releasing hormone (GnRH) in the placenta (Gibbons et al., 1975; Khodr ɔiler Khodr, 1980; Seppala et al., 1980). The placental content and concentration of H seem to vary with the gestational age, attaining maximal concentration in early tion and declining in mid and late gestation (Siler Khodr and Khodr, 1978). ιetic luteinizing hormone (LHRH) has been shown to ιlate release of immunoreactive α- and β-subunit of human chorionic gonado- ɪ(hCG) in vitro by human placenta in a dose dependent manner (Khodr and Siler- lr, 1978). A stimulation in circulating chorionic gonadotropin (CG) levels by H has also been observed in bonnet monkey by Rao et al. (1984).

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Recently Siler-Khodr *et al.* (1984) have reported an increased circulating levels of maternal GnRH during pregnancy which parallels placental concentration of GnRH. Abnormally low levels of circulating maternal GnRH preceded premature labour. These findings are indicative of placental dysfunction caused by low GnRH levels. We had earlier reported that repeated administration of a potent LHRH agonist or administration of monoclonal antibodies to LHRH around periimplantation period in baboons is able to curtail the pregnancy specific rise in circulating CG and progesterone, leading to termination of pregnancy (Das and Talwar, 1983; Das *et al.*, 1985a, b).

In this communication, we describe the effect of LHRH on CG and progesterone production *in vitro* by human placental villi from early pregnancy and also the ability of bioneutralizing monoclonal antibody against the decapeptide to block the action of LHRH on the placenta.

Materials and methods

Culture conditions

Placental tissue taken out by suction procedure from normal uncomplicated pregnancies of 8–10 weeks gestation, from subjects undergoing medical termination of pregnancy was collected in sterile conditions from our hospital in Delbecco's modified saline, pH 7·4. Villi were separated out carefully from the attached chorionic membranes, washed thrice with the culture medium (Dulbecco's Modified Eagle Medium) and were cut into small pieces (5–10 mg approx.). Each piece was placed randomly in 5 ml sterile glass culture tube containing 1 ml medium and was preincubated in an atmosphere of 95 % air $+ 5$ % CO_2 at 37°C for 24 h. In the initial experiments, cultures were further maintained upto 7 days with daily change of medium. Since the maximum response was obtained after the first day of culture, in all subsequent experiments cultures were maintained for 24 h only after which culture fluid was collected and stored at $-20°C$ until analysis.

Quantitation of hormones

Estradiol and progesterone were estimated directly from the fluid by the method of Aso *et al.* (1975) with the modification that the antigen and antibody were incubated for 18 h in cold rather than 30 min at 30°C. Quantitation of hCG in the medium was carried out by radioimmunoassay as described by Salahuddin *et al.* (1976).

Monoclonal antibody

Mouse hybrid cell clones secreting anti-LHRH monoclonal antibody were developed by fusion of SP2/O-Ag 1·4 myeloma cells with splenocytes of mouse immunized with LHRH tagged to tetanus toxoid (Gupta and Talwar, 1983). The monoclonals secreted from one of the clones $P_8 16_{62}$ reacted optimally with the native hormone. The association constant (K_a) for binding with LHRH was $1·2 \times 10^9$ L/mol. The monoclonal anti-LHRH antibody was competent to neutralize the *in vivo* bioactivity of the releasing hormone, as evidenced by block of ovulation and termination of pregnancy in mice (Das *et al.*, 1985a, b). In the placental cultures, the monoclonals were used at an antigen binding capacity of 3 μg/ml final dilution.

Results

Kinetics of LHRH response

After an initial pre-incubation of 24 h placental villi were cultured for 7 days with change of medium every 24 h. The daily release of hCG was determined over the total period of culture (figure 1). A significant increase ($P < 0.01$ to 0.05) in the gonadotropin secretion was observed upto 6 days in the treated set with about 3 fold stimulation after the first day of culture. A progressive decline in LHRH stimulated hCG secretion followed from second day onwards, till the sixth day. There was no difference between the control and experimental set on day 7 of culture. The concentration of estradiol measured at various time intervals was at the lowest detectable range of the assay in both control and experimental sets.
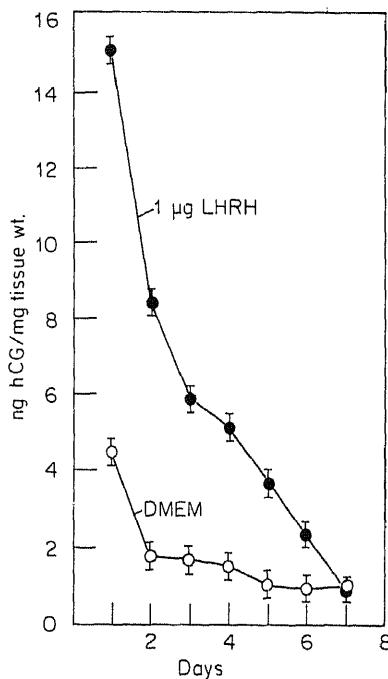
Figure 1. Secretion of hCG by the 10 week placenta in culture with or without LHRH. Each point is the mean of triplicates.

Dose response of LHRH on hCG and progesterone secretion

The effect of increasing concentration of LHRH on hCG and progesterone secretion by the placental villi were carried out in cultures maintained for 24 h (figures 2 and 3). Secretion of both the hormone in the medium was increased with LHRH and a maximum stimulation was observed with 1 μg concentration of the decapeptide ($P < 0.01$). Higher amounts of LHRH distinctly inhibited the gonadotropin as well as
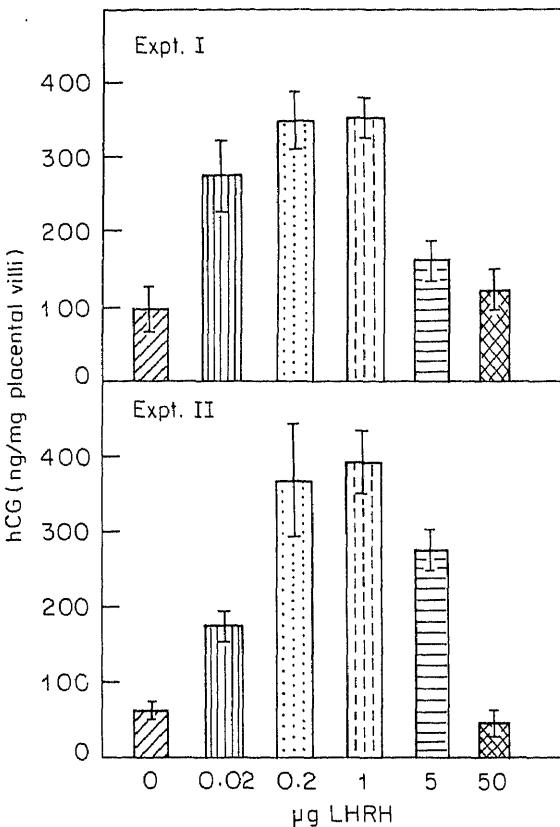
Figure 2. The effect of increasing concentration of LHRH on hCG secretion by the 10 week old placenta in culture. Results are the mean of quadruplicate sets. The bars represent the standard error of the mean.

the steroid secretion. Almost complete inhibition of secretion was observed at a concentration of 50 μg LHRH.

Effect of monoclonal antibodies to LHRH

Monoclonal antibodies at a dilution of 10^3 to 10^5 were added to cultures in the presence or absence of the releasing hormone. The antibody by itself did not have any effect on the basal secretion of the gonadotropin but was successful in inhibiting the LHRH induced increase in hCG and progesterone secretion (figures 4 and 5). The monoclonals completely suppressed the hormone secretions upto a dilution of 10^4.

Discussion

During pregnancy, the placenta develops into a highly active endocrine organ secreting large amounts of protein and also steroid hormones. Many of these hormones resemble
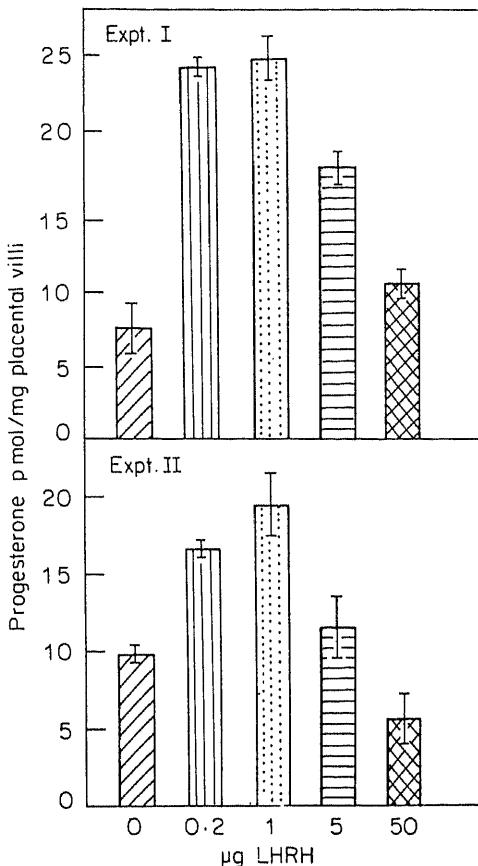
Figure 3. Dose response of LHRH on progesterone secretion by the 10 week old placenta in culture.

those secreted by the pituitary and the ovaries. The factors which control the secretion of pituitary and ovarian hormones are well understood. In contrast, very little is known about the regulation of placental hormones.

The human placenta was considered to be autonomous in its secretion of hormones until Gibbons *et al.* (1975) reported that placenta, chorionic or amniotic membrane extracts contained a biologically active LHRH. In addition, they determined the incorporation of radioactive amino acids into a molecule which resembled LHRH in its mobility on carboxy methyl cellulose chromatography. Later, Khodr and Siler-Khodr (1978) reported an increase in hCG in response to LHRH. They also measured the placental content of LHRH throughout gestation and found that the concentration of the releasing hormone is higher in the villous tissue than in the maternal or fetal circulation or amniotic fluid.

Using *in vitro* culture system, a reliable and repeatable method for the study of hCG secretion, we have observed a stimulation of the gonadotropin secretion by the
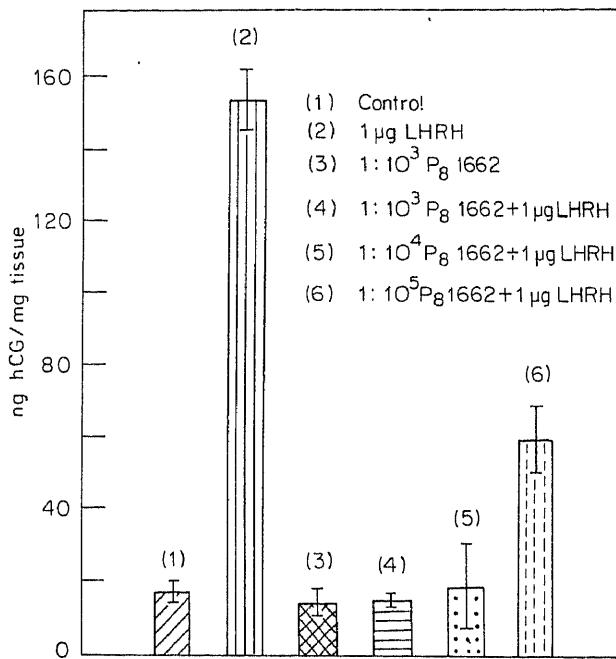
Figure 4. The inhibitory effect of anti-LHRH monoclonal antibodies ($P_8 16_{62}$) on LHRH induced hCG secretion by 8 week placenta in culture.
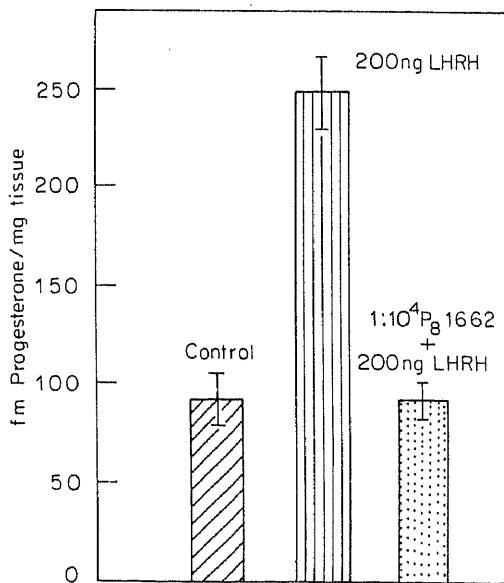
Figure 5. Effect of anti-LHRH monoclonal antibodies ($P_8 16_{62}$) on LHRH induced progesterone secretion by 10 week placenta in culture.

releasing hormone. A more pronounced and prolonged response to exogenous LHRH on hCG and progesterone production was noted when the villi were preincubated for 24 h before addition of the peptide in the culture. Analysis of the medium after the preincubation showed considerable amount of LHRH released. Addition of LHRH after the change of medium stimulated both hCG and progesterone production maximally on the first day of culture. The response declined progressively with each day of culture. The stimulation could be observed upto the sixth day in contrast to only second day of culture as reported for hCG by Khodr and Siler-Khodr (1978).

Higher concentrations of the releasing hormone inhibited both hCG and progesterone secretion. This may have been due to the well known desensitization or down regulation of the receptors by LHRH. Using term placenta Siler-Khodr and Khodr (1983) did not observe such on inhibition. This may be due to the differential state of the receptors in the placenta of 8-10 weeks gestation used in our studies to that of term placenta.

To further check the specific action of LHRH on placental function, we have used monoclonal antibodies against LHRH. Inhibition of the action of a hormone by antibodies is a classical technique in studying its physiological role. The block in the secretion of hCG and progesterone by anti-LHRH monoclonal antibodies indicate a specific response of the peptide on the placental functions. It also suggests that the affinity of LHRH for its receptors on the tissue may have been less than its affinity for the antibodies used in these studies. Since the affinity of the monoclonals for LHRH was in the order of 10^9, it appears that in contrast to the high affinity receptors on the pituitary, placenta probably contains low affinity receptors for LHRH. It may also be possible that the antibodies are able to bind to LHRH occupying receptors in the plasma membrane and block its action (Sternberger and Petrali, 1975; Sternberger *et al.*, 1978).

In summary, under the *in vitro* conditions, LHRH stimulates the secretion of hCG and progesterone. Higher amounts of the releasing hormone inhibit the secretion of both the hormones. Monoclonal antibodies against the decapeptide are able to block the action of LHRH on the placenta. This may be the mechanism by which repeated administration of LHRH agonists or anti LHRH antibodies bring about termination of early pregnancy in baboons.

Acknowledgement

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Prostaglandin-synthetase activity in developing toad ovary—
I. Detection and properties

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Abstract. Prostaglandin-synthetase activity has been measured in the microsomal fraction of developing toad (*Bufo melanostictus*) ovary using arachidonic acid as the substrate. Indomethacin (0·74 μM) and aspirin (0·35 μM) inhibit this activity. The activity is maximum in immature ovary and its level gradually decreases with maturity of the organ till the breeding season arrives, when it rises again. Time course study shows that the activity *in vitro* becomes steady after 3 min of incubation in all the cases, except the immature ones in which it sharply declines. Soluble supernatant was found to contain some inhibitory factor(s), which is partially inactivated by heating at 100°C for 5 min ($\sim$ 43%). Intraperitoneal injection of equine luteinizing hormone stimulates this enzyme activity in the mature ovary during non-breeding season. This suggests that similar to mammalians prostaglandin-synthetase, the toad ovary enzyme is also regulated by luteinizing hormone.

Keywords. Prostaglandin-synthetase; inhibitors; luteinizing hormone; developing toad ovary.

Introduction

Prostaglandin (PG)-synthetase, an enzyme involved in the conversion of all *cis*-eicosatrienoic acid and arachidonic acid to prostaglandin E_1 and E_2, has been widely detected in the various organs of the invertebrate and mammalian species (Christ and van Dorp, 1972). Accessory sex organs are known to contain most of the prostaglandins (van Dorp *et al.*, 1964). Ram testis is the richest source (Bergström *et al.*, 1964) of PG.

Rat (Zor *et al.*, 1973), rabbit (Le Maire *et al.*, 1973) and hen (Christ and van Dorp, 1972) ovaries have been found to synthesise prostaglandin E and F derivatives. It has been suggested (Goldberg and Ramwell, 1975; Lindner *et al.*, 1974) that ovum maturation and follicular rupture might need PGE compounds directly or indirectly. Prostaglandins and its synthetase activity in amphibian ovary has not been reported so far.

The physiology of the amphibian differs from that of mammals and aves. For instance, the ovary of an amphibian develops a characteristic pigmentation during

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maturation. The transition stages during this process are recognised by the following pigments in toad ovary:

Yellow ovary ⟶ Yellow ovary ⟶ Brown ovary
with a few   with some
black spots   black spots
(a)   (b)   (c)
                                                        ↓
                                              Black ovary
                                    (Non-breeding)   (Breeding)
                                         season   season
                                          (d₁)   (d₂)
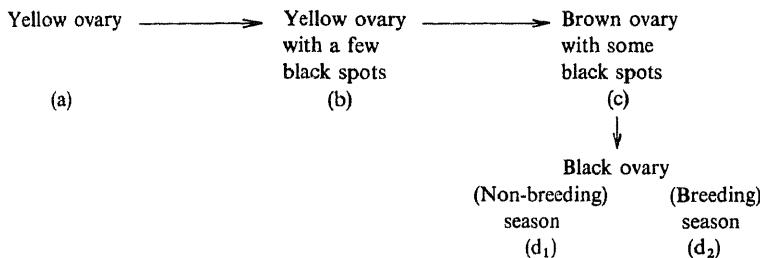
The dark pigment of the animal hemisphere of the amphibian ova has been found to be related to egg deposition in places exposed to sunlight. This is required for the protection of ova from UV-irradiation and atmospheric temperature (Salthe, 1969). The above changes in pigmentation might be related to biochemical processes in ovary. As PGE helps in the extrusion of ova from mature ovary (Walles *et al.*, 1975), its biosynthesis in the system could be taken into consideration.

The present paper describes the detection and properties of PG-synthetase in toad (*Bufo melanostictus*) ovary in the different stages of development.

Materials and methods

Materials

All chemicals and reagents used were of the highest commercial grade available. Arachidonic acid was a product of Nu-Chek Company, Elysian, Minnesota, USA. Glutathione, aspirin, L-epinephrine, luteinizing hormone (LH) from equine pituitaries and bovine serum albumin (BSA) were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Indomethacin, a drug with the trade name 'Indocin' was purchased from Merck, Sharpe and Dohme Research Laboratories. Prostaglandin E_1 and E_2 were kind gifts of Dr. J. E. Pike, Upjohn Company, USA.

Collection of samples

Whole ovary from *B. melanostictus* was collected from live animals at different stages of development and also from mature animal in their breeding and non-breeding seasons. External fat or any other attached tissue was removed. During development, the animals were maintained in their natural environment *i.e.* near a muddy pool with the regular diet white and red ants.

Tissue subfractionation procedure

Gonads were homogenised in 0·1 M phosphate buffer (pH 7·6), in Sorvall omnimixer (model no. 17106) for 10 min. The homogenate was centrifuged at 28,000 *g* for 30 min to remove the mitochondria. The postmitochondrial supernatant was centrifuged for

1 h at 1,05,000 g (Beckman L-5, rotor 50·2 ti) and the resulting microsomal pellet was used as the enzyme source. The soluble supernatant (designated as fraction S_{105}) was stored at $-20°C$ for further studies. All the operations were carried out at $4°C$.

Assay of prostaglandin synthetase

Prostaglandin synthetase activity was measured spectrophotometrically according to the method of Takeguchi *et al.* (1971). The optical density was measured in an EC-spectrophotometer (model no. GS-866C) at 580 nm.

The standard incubation mixture (4·85 ml) contained the following components: 82·5 μM arachidonic acid, 0·98 mM glutathione, 1 mM epinephrine and 80·5 μM Tris-HCl buffer (pH 7·8) and 100–130 μg of microsomal protein.

The product obtained after incubation at $30°C$ for 3 min, was extracted with ether and dried in vacuum. PGE formed was then estimated by Zimmerman reaction and measured at 580 nm. PGE formed was evaluated from the standard curve using PGE_1 as standard. Microsomal protein was measured at different stages of development of toad ovary (Lowry *et al.*, 1951) using BSA as standard.

Effect of indomethacin and aspirin

To the standard incubation mixture different doses of indomethacin and aspirin were added. The incubation mixture with drugs was preincubated for 5 min (Smith and Lands, 1971) before addition of arachidonic acid. Control experiments were also carried out with the drug in the absence of enzyme or substrate.

Effect of soluble supernatant (fraction S_{105}) on toad ovary

The fraction S_{105} was added to the incubation mixture at one fourth the total reaction volume. The supernatants (breeding season) from mature and immature ovaries were used. A portion of the soluble supernatant was dialysed overnight against 100 vol. of 0·1 M phosphate buffer (pH 7·6) at $4°C$. The soluble supernatant was boiled for 5 min and centrifuged at 3000 g. Protein content of all the fractions were measured (Lowry *et al.*, 1951) and a dose-dependent study was carried out. The incubation mixture containing the soluble supernatant was preincubated for 5 min at $30°C$.

Analysis of microsomal fatty acid of toad ovary by gas–liquid chromatography method

The total lipid was extracted from the microsomal pellet by the method of Bligh and Dyer (1959). Fatty acids were isolated from this lipid by saponification with 0·2 M NaOH and esterified with diazomethane (Schlenk and Gellerman, 1960). The fatty acid methyl esters (FAMES) were analysed by gas–liquid chromatography (GLC) (Pye Unicam, model 104 gas chromatograph with dual FID). The column (analytical 1·8 m × 2 mm) material used was 10 % polyethelene glycoladipate coated on a solid support 100–200 mesh diatomic CAW. Nitrogen at a flow rate of 60 ml/min was used as carrier gas at a column temperature of $180°C$. The FAMES were identified by comparing retention time with those of standard methyl esters and determining the carbon number of the component esters according to Ackman (1963).

Effect of LH on non-breeding season mature toad ovary

Equine LH (25 IU in 0·2 ml of 0·65 % saline) was injected intraperitoneally to the mature toads in the non-breeding season (Stage, d_1). Total ovary was collected after 4, 8, 16 and 20 h from different sets of animals and the PG-synthetase activity was measured. A control was set up by injecting the same amount of saline to the toad. Ovaries were collected from these control animals at the above time intervals and the PG-synthetase activity was measured as before. Each set contained 3 animals.

Results and discussion

Optimal conditions

Subcellular fractionation study of the toad ovary reveals that the PG-synthetase is exclusively a microsome-bound enzyme. Mitochondria and cytosol do not contain any PG-synthetase activity.

Toad, being a poikilothermic animal, the optimum temperature for PG-synthetase activity has been found to be 30°C (figure 1A). The time-course study (figure 1B) shows that the optimum time for maximal activity of this enzyme is 3 min and the rate of formation of prostaglandin is linear within this period. The optimum pH has been found to be 7·8 (figure 1C) which agrees with the reported value in other systems (Flower and Vane, 1974). A linear relationship between increasing amount of microsomal protein and also substrate with the amount of PG formed has been observed (figure 1D, E).

PG-synthetase activity at different stages of development of the ovary

Table 1 shows that the PG synthetase activity is markedly high in the immature ovary (Stage, a) and decreases gradually with the maturity of the organs becoming lowest in the fully mature ovary during the non-breeding season (Stage, d_1). However, the activity in the case of mature ovary in the breeding season (Stage, d_2) is 3 fold higher than that in the non-breeding season (Stage, d_1). This result is in accord with the findings in the rabbit ovary (Goldberg and Ramwell, 1975). Increased formation of PGE thus might help in the ovum maturation as well as follicular rupture for the extrusion of oocyte, as needed.

Effect of indomethacin and aspirin on PG-synthetase

Antiinflammatory drugs, such as indomethacin and aspirin are specific inhibitors of PG-synthetase (Vane, 1971; Flower, 1974). These drugs have shown a dose-dependent inhibition in the present case, thereby confirming the presence of PG-synthetase activity in the microsome of the developing toad ovary.

Indomethacin shows maximum inhibition at a concentration of 0·74 μM (figure 2A). This agrees well with the reported values (Smith and Lands, 1971; Sammuelsson *et al.*, 1975). The breeding season, non-breeding season and immature ovary enzyme at this concentration of indomethacin show 80·05, 95·2 and 86·36 % inhibition of activity, respectively. ID_{50} for these systems are 0·33, 0·33 and 0·25 μM respectively.
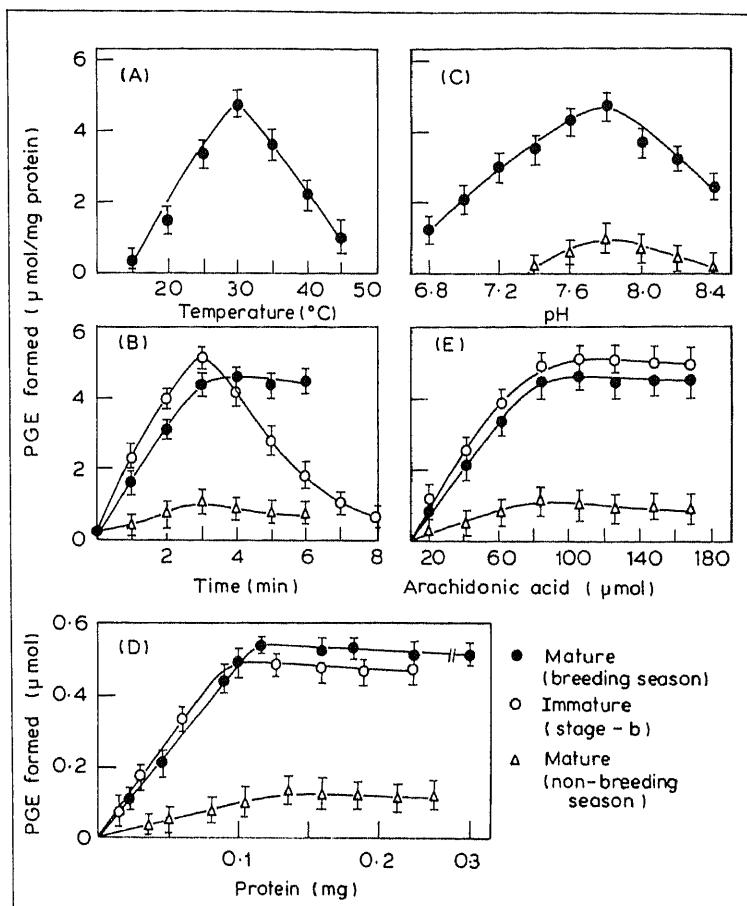
Figure 1. Optimum conditions for microsomal PG-synthetase activity in developing toad ovary.

The incubation mixture consisted of Tris-HCl buffer (pH 7·8), reduced glutathione (0·98 mM), L-epinephrine (1 mM), microsome as enzyme source (variable amount, D), arachidonic acid as substrate (variable amount, E). The temperature (A), pH (C), and time optima (B) with the standard assay condition have been determined.

Table 1. PG-synthetase activity in toad ovary.

Samples	μ mol of PGE formed/mg of protein/3 min
Stage, a	7·14 ± 0·17
Stage, b	5·28 ± 0·27
Stage, c	2·65 ± 0·15
Stage, d_1	1·10 ± 0·25
Stage, d_2	4·66 ± 0·33
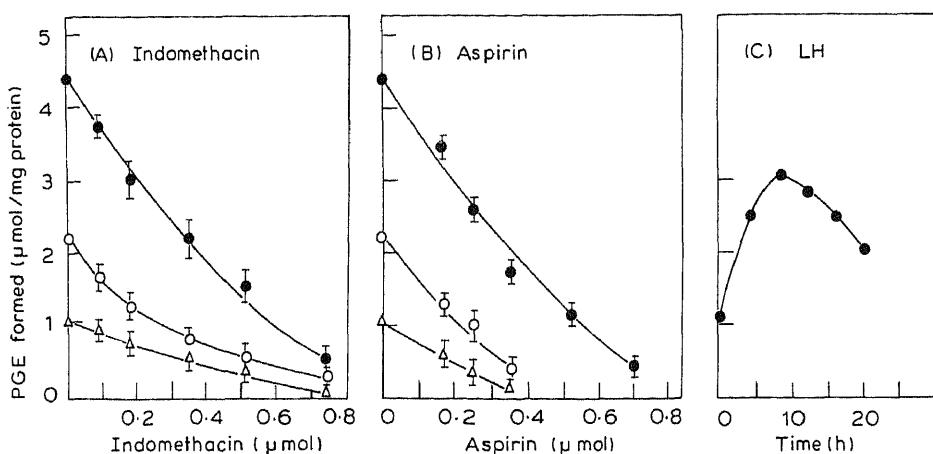
Figure 2. Effect of exogenous inhibitors and LH on PG-synthetase activity.
The reaction condition is the same as in figure 1. **A and B.** Dose-dependent inhibition by indomethacin and aspirin (△, mature non breeding; ●, mature breeding; ○, immature ovary). **C.** Effect of LH (●) on non-breeding toad ovary enzyme.

Aspirin has shown maximum inhibition at a concentration of $0.35 \, \mu$M. This concentration of aspirin inhibits PG-synthetase activity in immature ovary by 90%, in mature non-breeding season ovary by 81.4%, and in mature breeding season ovary by 62.4%. The ID_{50} values for these systems have been found to be 0.20, 0.33 and 0.17 μM respectively.

However, this concentration ($0.35 \, \mu$M) of aspirin needed to maximally inhibit toad enzyme is significantly lower than the value ($35 \, \mu$M) reported for other systems (Sammuelsson *et al.*, 1975). This difference may be due to the following reasons: most of the previous experiments have been carried out with crude homogenates of mammalian tissues. It is known that aspirin inhibits many other cellular functions besides PG-synthetase. Therefore, a higher level of aspirin may be utilized when a crude homogenate is used in the assay. The present experiment has been carried out with the microsomal fraction in which aspirin requirement is low. Here, all the experiments with aspirin have been done after 5 min of preincubation. The enzyme from all the stages of development responded to the same extent with this preincubation time (figure 2B), showing maximum inhibition at 0.35 μM. Smith and Lands (1971) have reported a time-dependent inhibition of the sheep vesicular gland deoxygenase system by aspirin and observed 80% inhibition after 5 min of preincubation with the drug. Similar preincubation effects have been observed with preparations from several other PG synthesizing tissues.

Effect of fraction S_{105} on PG-synthetase

Post microsomal supernatant of the bovine seminal vesicular gland and guineapig pulmonary system (Takeguchi *et al.*, 1971; Parkes and Eling, 1974) has been reported to contain some GP-synthetase inhibitors which have been identified as heat-labile protein or fatty acid (Takeguchi and Sih, 1973). PG-synthetase present in different

tissues has some common properties. Hence, the effect of the supernatant has also been studied in the present case.

Addition of a small amount of soluble supernatant (about 800 μg of protein) to the microsome results in a marked decrease in the enzyme activity. Table 2 shows 86·8 % inhibition in the enzyme preparations from immature ovary and 72 % in breeding season mature ovary. This suggests the presence of an inhibitor in fraction S_{105}. However, the activity is partially lost ($\sim$ 40 %) when soluble supernatant is boiled for 5 min. Thus, the inhibitors of the soluble supernatant appear to be mostly heat-labile, which may either be a protein or fatty acid. The protein concentration of the fraction S_{105} decreases (50 %) on boiling. The protein may be a fatty acid binding protein. Besides, the supernatant (S_{105}) has been found to contain free fatty acids which may also inhibit the PG-synthetase. Thus S_{105} may contain protein and/or fatty acid which might be responsible for the inhibition of PG-synthetase in the microsomal fraction.

It has already been described that the activity has been found to decrease with maturity of ovary till the non-breeding season and increase by 3-fold in the breeding season due to the physiological need of the system. The intriguingly high activity present in the immature ovary has not been hitherto explained. Herlitz (1974) and Lieberman *et al.* (1975) have observed stimulated lactic acid production and glucose oxidation when the immature ovary was incubated with PG. This may be a plausible explanation for more PGE formation in the immature ovary.

The level of PGE formed at different stages of developments may be regulated by (i) the metabolism of PGE compounds formed in the system, (ii) non-availability of the substrate and (iii) hormone.

The following experiments have been carried out to establish these possibilities.

Endogenous substrate availability

The fatty acid profile of the microsomal lipid has been analysed by GLC (table 3). It has been found that almost equal amounts of arachidonic acid (a substrate of PG-synthetase) are present in both the immature and mature ovary. Linoleic acid, a precursor of arachidonic acid and also an inhibitor of PG-synthetase (Pace Asciak and

Table 2. Effect of fraction S_{105} on PG-synthetase.

System	PG-synthetase activity (μ mol of PGE formed/ mg protein/3 min)	Inhibition (%)
A. *Mature breeding season ovary (stage, d_2)*		
(i) Microsome	4·66 ± 0·17	—
(ii) Microsome + S_{105}	1·30 ± 0·23	72·1
(iii) Microsome + boiled S_{105}	3·07 ± 0·25	34
B. *Immature ovary (stage, b)*		
(i) Microsome	5·28 ± 0·27	—
(ii) Microsome + S_{105}	0·69 ± 0·30	86·4
(iii) Microsome + boiled S_{105}	3·09 ± 0·25	41·4

Table 3. Content of microsomal arachidonic acid and linoleic acid.

	Fatty acid (%)	
Component	Mature ovary (Stage, d_1)	Immature ovary (Stage, b)
Linoleic acid (18:2)	13·43	14·08
Arachidonic acid (20:4)	15·63	13·51

Wolfe, 1968) is present in almost equal amounts in all the cases. Both esterified (in phospholipid and cholesterol ester) and non-esterified arachidonic acid present in the microsomal lipid may contribute to the *in vivo* PG-formation. It has been already established that endogenous arachidonic acid is made available to PG-synthetase *via* phospholipase action on the phospholipids of the system (Zor and Lamprecht, 1977). Arachidonic and linoleic acids do not seem to play a critical role in the regulation of PG-synthetase activity in developing ovary. Hence it is possible that hormones such as LH may have a direct role on PG-synthetase levels in ovary.

Effect of LH

LH induces ovum maturation (Lindner *et al.*, 1974), which is followed by follicular rupture. The level of LH has been found to increase during breeding season (Lofts, 1974). Several studies (Bauminger and Lindner, 1975; Johnsson *et al.*, 1975) show that the formation of PGE compounds may also be induced by LH by two pathways; one, by inducing *de novo* synthesis of PG-synthetase and second, by increasing the substrate availability. Therefore, the *in vivo* effect of the luteinizing hormone on the non-breeding season toad ovary enzyme has been studied here.

  Equine LH was administered to non-breeding season mature toad intraperitoneally. The PG-synthetase activity was measured at different time-intervals both in control and experimental samples. Figure 2C shows the difference in activity between control and the experimental tables at different time intervals. This shows that on administration of LH, the PG-synthetase activity gradually increases upto 8 h (two-fold more) and then decreases. This *in vivo* effect of LH on prostaglandin production in non-breeding season ovary might explain the high activity of PG-synthetase in the breeding season.

Acknowledgements

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DNA binding proteins of rat thigh muscle: Purification and characterization of an endonuclease

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Abstract. Two major DNA binding proteins of molecular weights 34,000 and 38,000 have been identified in the 30,000 g supernatant (S-30) fraction of rat thigh muscle extracts. The presence of 38 KD DNA binding protein in the muscle S-30 could be demonstrated only if Triton X-100 treated extracts were used for affinity chromatography suggesting that this protein may be a membrane associated DNA binding protein. The 38 KD DNA binding protein differed from the 34 KD DNA binding protein also in its chromatographic behaviour in DE-52 columns in which the 38 KD protein was retained, while the 34 KD protein came out in the flow-through in an electrophoretically pure form. The 34 KD DNA binding protein can also be purified by precipitation with $MgCl_2$. Incubation of 0·15 M NaCl eluates (containing the 38 KD and/or 34 KD DNA binding protein) in the presence of 100 mM Mg^{2+} resulted in the specific precipitation of the 34 KD protein. Prolonged incubation (30 days) of the 0·15 M NaCl eluates containing the two DNA binding proteins at 4°C led to the preferential degradation of the 34 KD DNA binding protein. Nitrocellulose filter binding assays indicated selective binding of purified 34 KD protein to ss DNA. Purified 34 KD DNA binding protein cleaved pBR 322 supercoiled DNA, and electrophoresis of the cleavage products in agarose gels revealed a major DNA band corresponding to the circular form of DNA.

Keywords. Affinity chromatography; DNA binding protein; endonuclease cleavage of pBR 322 DNA.

Introduction

As a result of the realization of the importance of DNA endonucleases in DNA metabolism, especially in the processes of initiation of DNA synthesis and repair, several attempts have been made to isolate and characterize these proteins. Affinity chromatography of proteins on DNA-cellulose columns has proven to be a valuable technique for the purification of endonucleases and other DNA binding proteins. Restriction enzymes and other endonucleases bind to double-stranded regions or to localized single-stranded stretches of double-stranded and superhelical DNAs while S-1 nuclease binds to single-stranded DNAs. Although numerous endonucleases have been isolated from prokaryotic cells and their biological functions established, investigations on eukaryotic endonucleases were of recent origin. DNA-binding

Abbreviations used: DBP, DNA binding protein; βME, β-mercaptoethanol; PMSF, phenyl methyl sulphonyl fluoride; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

proteins with topoisomerase activities have been reported to be present in rat liver (Mc Conaughy *et al.*, 1981; Ross *et al.*, 1983). Machray and Bonner (1981) have shown the conversion of super-helical pBR 322 DNA into linear form by a chromatin associated DNA binding protein (DBP). A DBP with endonucleolytic activity specific for apurinic and apyrimidinic DNA has been isolated from human placenta (Shaper *et al.*, 1982). Another endonuclease from placenta was shown to cleave pBR 322 DNA to linear form (Premeela *et al.*, 1984).

A 34 KD DBP was found to be present in large quantities in Yoshida ascites tumour cells (Rajakumar and Shanmugam, 1983). Since this tumour is of connective tissue origin, an attempt was made in search of similar DBPs in normal connective tissues. The results presented here show the presence of two DBPs of molecular weight 38,000 and 34,000 in rat thigh muscle of which the latter protein has been purified and studied in detail. The purified 34 KD protein is shown to possess endonuclease activity capable of converting supercoiled pBR 322 DNA into circular form. The muscle enzyme differs from the above mentioned eukaryotic endonucleases in that it produces a single cut circular DNA as a major cleavage product.

Materials and methods

Calf thymus DNA, Sigma cell (R) type-100 cellulose, phosphorylase a, bovine serum albumin, human γ-globulin, ovalbumin, cytochrome C, Tris and Dowex were the products of Sigma Chemical Co., St. Louis, Missouri, USA. Acrylamide and bis-acrylamide were the products of Bio-Rad. DEAE-cellulose (DE-52) and phos-phocellulose were obtained from Whatman, Co, UK. Nitrocellulose membrane filters were the products of Schelicher and Schuell inc., Germany. $[^{32}P]$-orthophosphoric acid (carrier-free) was obtained from Bhabha Atomic Research Centre, Bombay.

Isolation of $[^{32}P]$-labelled Escherichia coli DNA

$[^{32}P]$-DNA was extracted from *E. coli* using phenol–chloroform–isoamylalcohol according to the modified method of Marmur (1961). The isolated DNA was dissolved in 1 mM NaCl and passed through a column of Dowex-1 × 4 to remove anionic contaminants. The flow-through was collected and precipitated with ethanol. The precipitate was dissolved in 1 mM NaCl for use in further studies. DNA was denatured by heating at 100°C for 10 min followed by rapid cooling.

Preparation of cytoplasmic fractions

All operations were carried out at 0–4°C unless otherwise mentioned. For the preparation of muscle extract, thigh muscle of albino rats was minced and then suspended in 10 volumes of sterile water. The suspension was homogenized thrice in a Sorvall homogenizer with the speed setting at 5, for 30 sec each time. The suspension was made 20 mM Tris pH 8·1, 1 mM EDTA, 1 mM β-mercaptoethanol (βME) and 1 mM phenyl methyl sulphonyl fluoride (PMSF). The homogenate was centrifuged at 30,000 *g* and the supernatant was used for the analysis of DBPs.

In some preparations, 1 % Triton X-100 was used in the extraction procedure for the preparation of S-30.

DNA-cellulose chromatography

Denatured DNA-cellulose column chromatography was carried out as described earlier (Shanmugam *et al.*, 1975; Rajakumar and Shanmugam, 1983, 1984). Muscle S-30 containing approximately 60–70 mg protein was chromatographed in a 0·8 × 4 cm denatured DNA-cellulose column. After washing the column extensively with buffer A (20 mM Tris pH 8·1, 1 mM EDTA, 1 mM βME and 1 mM PMSF) containing 50 mM NaCl, the bound proteins were eluted with the above buffer containing 0·15 M NaCl.

DEAE-cellulose chromatography

The 0·15 M NaCl eluate of DNA-cellulose column was dialysed extensively against buffer A containing 50 mM NaCl for 12 h with two changes of buffer. A column (1·5 × 8 cm) was packed with pre-equilibriated DEAE-cellulose and then washed with 100 ml of the column buffer containing 50 mM NaCl. The dialysed 0·15 M NaCl eluate from DNA-cellulose column was loaded onto the column at the flow rate of 10 ml/h. The flow-through was collected separately. The column was washed with 50 ml of column buffer and this was pooled with the flow-through. The bound proteins were then eluted using the column buffer containing 0·15, 0·4 and 2 M NaCl.

The pooled flow-through and the wash fractions were concentrated by dialysis against sucrose and then precipitated with 100 % ammonium sulphate. The precipitate was collected by centrifugation at 30,000 g for 15 min and dissolved in a minimal quantity of column buffer.

Filter binding assay

Nitrocellulose filter binding assay was performed according to the procedure of Tsai and Green (1973). Before use, the filters were boiled in water and then soaked in filter binding buffer (20 mM Tris-HCl, pH 8, 1 mM EDTA, 2 mM βME and 5 % glycerol) for at least 12 h. For a typical assay, 2 μg of [^{32}P]-*E. coli* DNA (1,200 cpm) and appropriate concentrations of protein, in a final volume of 100 μl, was incubated at 37°C for 30 min. The assay mixture was slowly filtered through prewashed filters at a flow rate of 0·5 ml/min. The filters were washed thrice with 3 ml of buffer containing 1 % dimethyl sulphoxide, dried and counted.

Endonuclease assay

Endonuclease activity was monitored according to the modified procedures of Clough (1979). The assay mixture (20 μl) contained 2 μl of enzyme sample, 5 mM MgCl$_2$, 5 μg of pBR 322 DNA, 10 mM βME and 50 mM Tris pH 8·3. All samples were incubated at 37°C for 30 min. The reaction was stopped by heating the samples at 60°C for 5 min. After the addition of glycerol (10 %) and bromophenol blue (0·002 %), the samples were loaded on 1 % agarose gels and electrophoresed (Premeela *et al.*, 1984).

Electrophoresis of proteins

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out as described earlier (Rajakumar and Shanmugam, 1983).

Results and discussion

DNA-cellulose chromatography of muscle proteins

DNA-cellulose chromatography of muscle S-30 extracts on ss DNA-cellulose columns and subsequent elution of bound proteins with 0·15 M NaCl removed most of the DBPs from the column. The 0·15 M NaCl eluate contained 2·5 % of the total proteins used for chromatography. Figure 1 shows a gel electrophoretic profile of DBPs present in the 0·15 M NaCl eluate. A 38 KD and a 34 KD protein were the major DBPs in the 0·15 M NaCl eluates obtained from the denatured DNA-cellulose column (figure 1, lane C). These two DBPs were observed only if a Triton X-100-treated S-30 was used for chromatography. As seen in figure 1, lane D, when the cytoplasmic fraction was prepared without using Triton X-100, only the 34 KD protein was seen in the 0·15 M
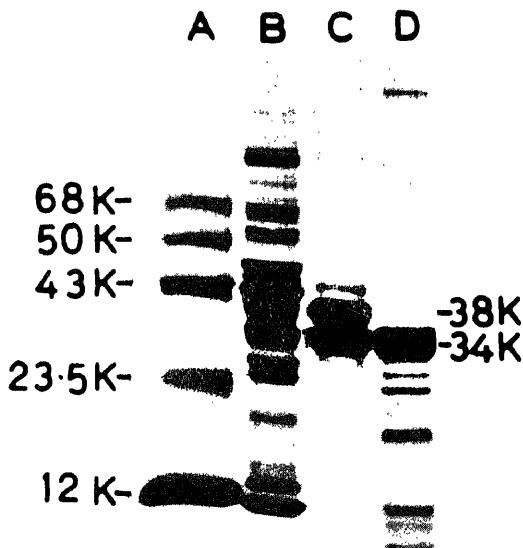

Figure 1. SDS-PAGE of muscle DBPs. Muscle extract was prepared with or without using Triton X-100 and chromatographed on heterologous denatured DNA–cellulose columns. The bound proteins were eluted and electrophoresed in 10–18 % polyacrylamide gradient gels. Lane A, molecular weight markers; lane B, S-30 prepared using Triton X-100; lane C, DBPs eluted with 0·15 M NaCl from denatured DNA–cellulose column in which S-30 prepared in the presence of Triton X-100 was chromatographed; lane D, DBPs, eluted from denatured DNA–cellulose column in which S-30 prepared in the absence of Triton X-100 was chromatographed.

NaCl eluates of denatured DNA-cellulose columns. These results suggest that the 38 KD DBP may be a membrane-associated protein.

Effect of Mg^{2+} on the muscle DBPs

When the 0·15 M NaCl eluate containing the 34 KD and 38 KD proteins were incubated with 100 mM Mg^{2+} at 37°C for 18–24 h, a milky white precipitate was formed. After centrifugation and electrophoresis, the precipitate was found to contain the 34 KD DBP and was nearly 95 % pure (figure 2). Similar results were obtained when 0·15 M NaCl eluate containing only the 34 KD DBP was used for Mg^{2+} precipitation.

Incubation of the DBPs with Mg^{2+} at 4°C for longer periods (1 month) resulted in the preferential degradation of the 34 KD DBP to smaller polypeptides (figure 2B).

Purification of the muscle 34 KD DBP

In the purification profile shown in figure 3, 0·15 M NaCl eluates of the ss DNA-cellulose columns (in which muscle extracts prepared in the presence of Triton X-100 were chromatographed) were used. These eluates contained both the 34 KD and 38 KD DBPs. When these eluates were chromatographed on DEAE-cellulose columns, the flow-through had the 34 KD DBP in a pure form (figure 3). Similarly, when the 0·15 M NaCl eluate containing mainly the 34 KD DBP (obtained from extracts prepared in the
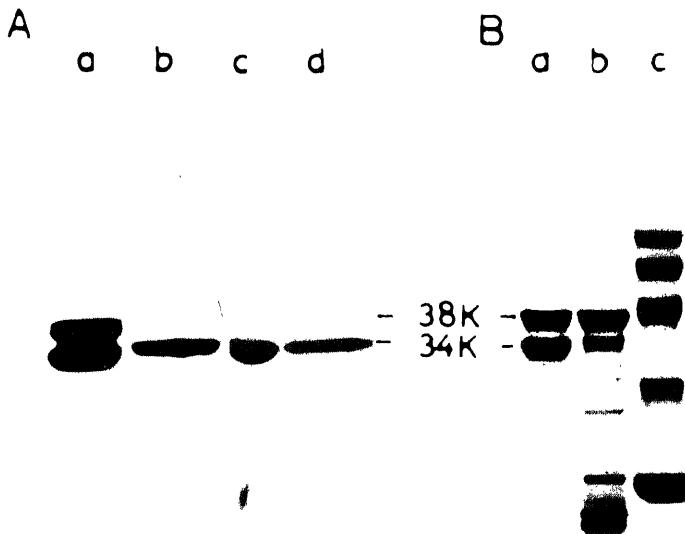

Figure 2. Mg^{2+} precipitation of DNA-binding proteins. The 0·15 M NaCl eluates from ss DNA–cellulose columns were adjusted to 100 mM Mg^{2+} using $MgCl_2$ and were incubated at 37°C for 18–24 h. A. Lanes a and c are unincubated controls; lanes b and d, Mg^{2+}-precipitated samples. The DBPs used for lanes a and b were obtained from Triton X-100 treated extracts while the DBPs used for lanes c and d were derived from S-30 prepared in the absence of the detergent. B. Shows the effect of prolonged (1 month) incubation of DBPs in 100 mM Mg^{2+} at 4°C. Lane a, unincubated 0·15 M NaCl eluate and lane b, incubated sample. For this experiment, the DBPs were obtained from Triton X-100 treated S-30, c, Molecular weight markers.
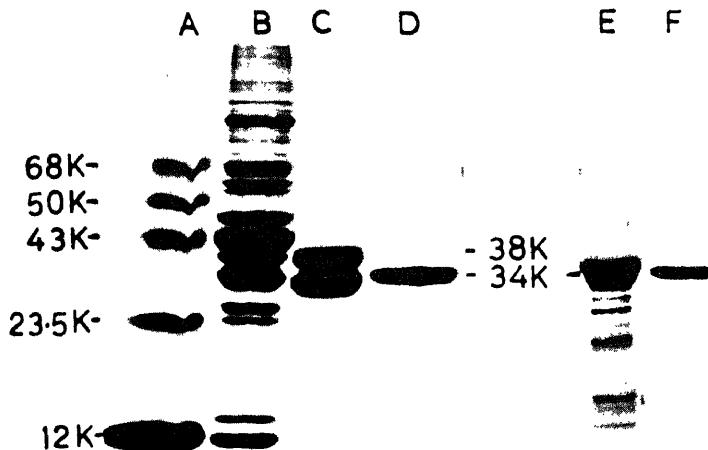
Figure 3. Purification of 34 KD DBP. Lane A, molecular weight markers; lane B, total proteins of muscle extract prepared using Triton X-100; lane C, 0·15 M NaCl eluate from ss DNA–cellulose column in which S-30 prepared in the presence of Triton X-100 was chromatographed; lane D, unbound fraction from DEAE–cellulose column in which the 0·15 M NaCl eluate shown in lane C was chromatographed; lane E, 0·15 M NaCl eluate from ss DNA–cellulose column in which muscle extracts prepared in the absence of Triton X-100 was chromatographed; lane F, unbound fraction from DEAE–cellulose column in which the 0·15 M NaCl eluate shown in lane E was chromatographed. The protein fractions were electrophoresed in 10–18 % polyacrylamide gradient gels.

absence of Triton X-100) was chromatographed, the flow-through contained the 34 KD DBP in an electrophoretically pure form. In order to visualize the impurities, the gels were over-loaded with the purified proteins. Over-loading did not reveal the presence of contaminant proteins, suggesting that these proteins are in a highly pure form (figure 3, lanes D and F). Our techniques, however, do not rule out the possibility of the presence of contaminant proteins with the same molecular weights. Analysis of the purified proteins in 2-dimensional gels will enable a rigorous establishment of the purity of these proteins.

The results presented above indicate that the 38 KD protein differs from the 34 KD DBP in two aspects: (1) the former protein could not be precipitated by Mg^{2+}, while the latter could be precipitated, and (2), the 38 KD protein was retained on the DEAE-cellulose column while the 34 KD protein was not retained.

Specificity of binding

The binding specificity of the 34 KD DBP to either native or denatured DNA was determined using nitrocellulose filter binding assay according to the procedures of Tsai and Green (1973). Native or denatured DNA (2 μg) was incubated with increasing amounts of the 34 KD DBP for 30 min and assayed. The results presented in figure 4A indicate that the muscle 34 KD DBP preferentially bound to ss DNA. The protein
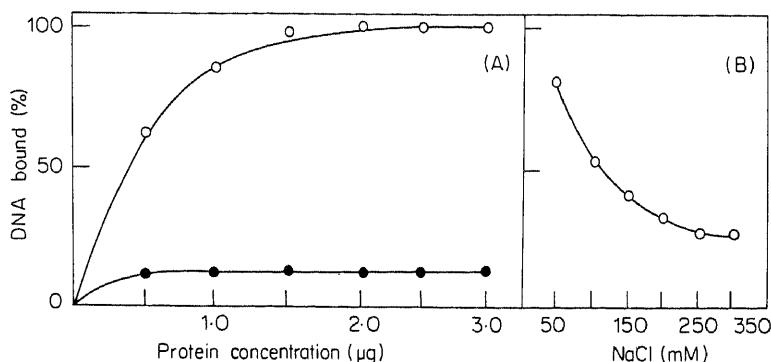
Figure 4. Nitrocellulose filter binding assay of 34 KD DBP. **A.** Increasing quantities of the muscle 34 KD DBP were incubated with 2 μg of native (●) or denatured (O) [^{32}P]-DNA (1200 cpm) from *E. coli*. The DNA–protein complexes were separated by filtration on nitrocellulose membrane filters and counted in aqueous scintillation fluid. **B.** Effect of increasing concentrations of NaCl on the binding of the muscle 34 KD DBP. Filter binding assay was done with 1·5 μg of 34 KD DBP, 2 μg of heat denatured [^{32}P]-DNA (1200 cpm) from *E. coli* and indicated amounts of NaCl.

retained 10 % of the native DNA and 95 % of the ss DNA. The saturation levels of the muscle DBP were 0·5 μg and 1·5 μg for native and denatured DNAs respectively.

In terms of preferential binding to ss DNA, this protein seems to be similar to the 33,000–34,000 molecular weight DBPs of calf thymus, human placenta and Yoshida ascites sarcoma (Herrick and Alberts, 1976; Premeela *et al.*, 1984; Rajakumar and Shanmugam, 1983, 1984). Other eukaryotic DBPs of similar molecular weights were shown to bind both to ss and ds DNAs (Novak and Baril, 1978; Mather and Hotta, 1977).

Effect of ionic strength on binding

The effect of increasing concentration of ions on the binding of DBP to DNA, was determined by altering the ionic concentrations in the incubation medium. The results shown in figure 4B indicate that increasing salt concentrations decreased the complex formation between the DNA and protein.

Endonuclease activity of 34 KD DBP

Recent studies have shown that different types of endonuclease activities have been found to be associated with DNA binding proteins. Therefore, it was of interest to see whether the purified 34 KD DBP of muscle possessed any endonucleolytic activity. For endonuclease assays, pBR 322 DNA was used as substrate and the cleavage products were analysed by electrophoresis in agarose gels. The results presented in figure 5, lane A indicate the presence of 3 bands corresponding to multimeric, circular and superhelical forms in the control samples incubated in the absence of the 34 KD protein. However, DNA samples incubated with the 34 KD protein showed a steep increase in the amount of the circular form with concomitant decrease in the supercoiled form, (figure 5, lanes B and D). These results suggest that the 34 KD
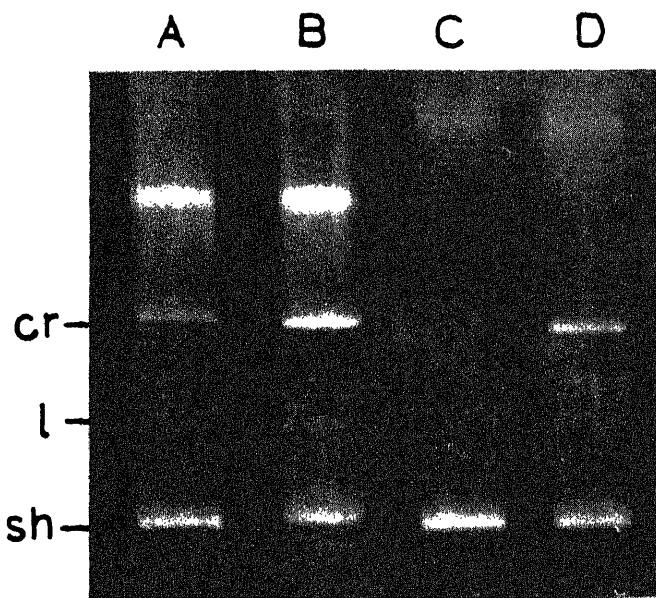
Figure 5. Endonucleolytic cleavage of native and heat denatured pBR 322 DNAs by muscle 34 KD DBP. Native (lane B) and denatured (lane D) pBR 322 DNA (5 μg) were incubated with 0·5 μg of muscle 34 KD DBP for 30 min at 37°C. Lanes A and C respectively contained native and denatured pBR 322 DNAs incubated in the absence of 34 KD DBPs. After the assay, the samples were electrophoresed in 1 % agarose gels for 5 h at 60v. m, multimer; Cr, relaxed circular; l, linear; Sh, super helical.

protein possesses endonucleolytic activity capable of converting the super-helical DNA into circular form.

When the endonuclease activity was studied as a function of increasing time (figure 6), for a period of 15, 30, 45 and 60 min, the following results were obtained: (i) the conversion of supercoiled DNA into the circular form gradually increased with the time of incubation, (ii) the optimal time for maximal enzyme activity was 30 min; and (iii) no additional degradation products were seen following longer periods of incubation.

Endonuclease activity increased with the increase in protein. 3 μg of the 34 KD protein was able to completely convert the pBR 322 DNA into the circular form. No additional degradation products were seen when increased amounts of protein were used (figure 7).

The results presented in figures 5–7 show that the 34,000 molecular weight DBP possesses endonuclease activity. In contrast to the action of other eukaryotic endonucleases which produced linear DNAs from supercoiled DNAs (Machray and Bonner, 1981; Premeela et al., 1984), the muscle enzyme produced circular DNAs from superhelical pBR 322 DNA suggesting that the endonuclease activity cleaved the DNA by introducing a single cut in one of the strands of the DNA. The fact that increased time of incubation (figure 6) or higher concentrations of DBP (figure 7) failed to
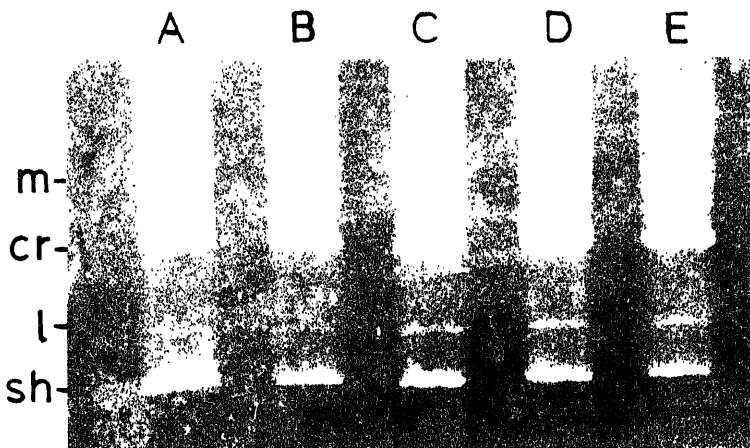
Figure 6. Time course of cleavage of native pBR 322 DNA by muscle 34 KD DBP. Aliquots containing 5 μg pBR 322 native DNA were incubated with 1 μg of the purified protein for the desired periods and electrophoresed in 1% agarose gels for 5 h at 60v. Lane A, control pBR 322 DNA, lanes, B, C, D and E contained pBR 322 DNAs incubated with muscle 34 KD DBP for 15, 30, 45 and 60 min respectively.
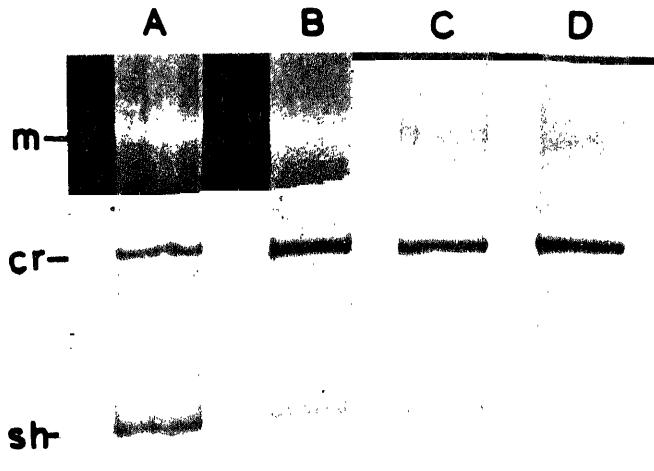

Figure 7. Effect of increasing concentrations of the DBPs on endonuclease activity. Aliquots containing 5 μg of pBR 322 DNA were incubated with increasing concentrations of muscle 34 KD DBP for 30 min and electrophoresed in 1% agarose gels. Lane A, control pBR 322 DNA; lanes B, C and D contained samples incubated with 1, 2 and 3 μg of proteins respectively.

produce additional bands other than the single-nick circular form, indicates the absence of contaminating nucleases.

In the light of the implication of the role of DNA endonucleases in the initiation of DNA replication and repair (Cleaver, 1974; Linn, 1982; Brown *et al.*, 1982), it will be of interest to know the function of the muscle endonuclease in DNA metabolism. Studies

aimed at a detailed characterization of the muscle endonuclease, including the determination of cleavage site(s) are in progress.

Acknowledgements

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The nature of lectins from *Dolichos lablab*

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Abstract. The lectins from the seeds of *Dolichos lablab* var. *lignosus* (field bean) and *Dolichos lablab* var. *typicus* (lablab bean) have been isolated in a homogeneous form by affinity chromatography on D-mannose linked Sepharose. Both the lectins are glycoproteins and have a molecular weight of 60,000 and $S_{20,w}$ value of 5·2 and seem to be made up of 4 similar subunits (apparent molecular weight 15,000). The carbohydrate content of the lectins is mostly fucose (2–5 mol per mol of protein), mannose (5–8 mol per mol of protein) and N-acetyl glucosamine (1–2 mol per mol of protein). The amino acid composition of both the lectins was similar and methionine and half cystine could not be detected. Both the lectins have similar tryptic peptide map. Alanine and serine were the only N and C-terminal amino acids for both lectins. The lectins were found to contain low amounts of bound metals such as manganese, magnesium and calcium. The near ultra-violet circular dichroism spectra of the lectins are similar to that of Sainfoin. Circular dichroism data indicate that tyrosine and tryptophan residues are involved in sugar binding.

The lectins are nonspecific for human blood groups and they agglutinate a variety of other erythrocytes. Among a number of sugars, D-glucose and D-mannose inhibited the haemagglutinating activity of the lectins. The lectins were antigenically similar.

Keywords. *Dolichos lablab*; lectin; field bean; lablab bean; physicochemical and biological properties.

Introduction

Lectins are cell agglutinating sugar specific proteins that are widely distributed in leguminous plants (Sharon and Lis, 1972; Bog-Hansen, 1981). The genus *Dolichos* (family leguminosae, subfamily paplionaceae) consists of a number of plants among which *Dolichos lablab* var. *lignosus* (field bean) and *Dolichos lablab* var. *typicus* (lablab bean) are grown fairly widely in India, Australia and some African countries for various food uses (The Wealth of India, 1952; Tropical legumes, Resources for the future, 1979; Duke, 1981). Unlike the lablab bean whose pods are used as a vegetable, the food form of the field bean is largely as seed. The lectin from the field bean seeds has earlier been purified by conventional methods of protein fractionation and some properties studied (Narayana Rao *et al.*, 1976; Hariharan, 1979).

As part of a general programme on the study of biologically important proteins from legumes used in the Indian dietary, the work on plant lectins has been initiated. It is

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Abbreviations used: CD, Circular dichroism; EDTA, ethylenediamine tetraacetic acid; M_r, molecular weight; SDS, sodium dodecyl sulphate; TPCK, N-tosyl-L-phenylalanylchloromethyl ketone; UV, ultra-violet; Con A, concanavalin A.

proposed to investigate the primary structure of these ubiquitous proteins to obtain information regarding their evolution in nature, establish finer differences in the molecular specificity of these proteins with respect to carbohydrates and explore their possible uses in separation of different cell types. The present study provides the basic information regarding the physico-chemical and biological properties of the field bean and lablab bean lectins. A preliminary report regarding some aspects of this work has been presented earlier (Siva Kumar *et al.*, 1983). Recently, Guran *et al.* (1983) have reported the purification and properties of the lectin from field bean, but our results are at considerable variance with their findings.

Materials and methods

Materials

Divinyl sulphone was a product of Fluka AG, Switzerland. Sepharose 6B, Concanavalin A Sepharose were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Dansyl chloride, sodium dodecyl sulphate (SDS) were purchased from Pierce Chemical Company. Biogel P-30, P-100 and P-200 were purchased from Biorad Laboratories, USA. Concanavalin A, pea lectin, lentil lectin, wheat germ lectin, *Dolichos biflorus* lectin, standard proteins, and all sugars used were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. N-Tosyl-L-phenylalanylchloromethyl ketone (TPCK) treated trypsin (EC 3.4.21.4) was obtained from Serva Fine Biochemicals, Heidelberg, Carboxy-peptidase A (EC 3.4.17.1) was purchased from Boehringer Co., Germany, Guanidine hydrochloride was purchased from Riedel-dehaan, W. Germany. All other reagents used were of analytical grade. Seeds of field bean (local variety Maniavare) and lablab bean (local variety Chikkaballapur) were obtained from the Department of Agricultural Botany, University of Agricultural Sciences, Hebbal, Bangalore. Human blood was obtained from the blood bank of the local hospital. Rabbit and guinea pig blood were obtained from the Animal House of the Institute. Sheep and fowl blood were obtained from the local slaughter house. Phosphate buffered saline, Pi/NaCl (0·006 M sodium/potassium phosphate buffer pH 7·4 containing 0·15 M NaCl) was used to wash the erythrocytes. Trypsinisation of erythrocytes was carried out on a 2 % cell suspension in Pi/NaCl using 0·1 % trypsin at 37°C for 1 h and the cells washed with Pi/NaCl or 0·15 M NaCl.

Methods

Preparation of affinity column: Mannose linked to Sepharose 6B *via* divinyl sulphone was prepared by the method described in literature (Fornstedt and Porath, 1975; Hapner and Robbins, 1979).

Protein estimation: The method of Lowry *et al.* (1951) using bovine serum albumin as standard was routinely used. For monitoring column effluents protein was determined spectrophotometrically by the method of Warburg and Christian (1941).

Carbohydrate determinations: The lectins were hydrolysed with 1 N HCl *in vacuo* for 2 h at 110°C and later processed for neutral and amino sugars on a Dowex-50-H$^+$

column (Boas, 1966). Neutral sugars were determined by the method of Dubois *et al.* (1976) using D-mannose as standard. Amino sugars were determined by a modified procedure of Elson and Morgan (Boas, 1966). Neutral sugars were identified as alditol acetates by gas liquid chromatography with inositol as the internal standard (Kannan *et al.*, 1974). Amino sugars were also identified on the LKB amino acid analyser under standard conditions recommended by the manufacturers using a citrate buffer sequence pH 3·2, 4·25, 6·45 at different temperatures on a ultrapac 11 cation exchange resin with particle size of 11 μm. The flow rate of the buffer was 40 ml/h and glucosamine was eluted at 64 min.

Amino Acid analysis: Amino acid composition of the lectins was determined on a Dionex-Durrum (Kit-MBF) automatic amino acid analyser as well as on a LKB Alpha 4150 amino acid analyser after acid hydrolysis with 6 N HCl at 110°C (Moore and Stein, 1963). Methionine sulphone and cysteic acid were determined after performic acid oxidation of the lectins and acid hydrolysis (Hirs, 1956). Tryptophan was determined by the spectrophotometric method of Edelhoch (1967). N-Terminal amino acid was detected by the procedure of Gray (1972). C-Terminal amino acid was determined using pancreatic carboxypeptidase A (Ambler, 1972).

Immunodiffusion: Antibodies against the purified lectins were raised by immunisation in rabbits according to standard procedures and the antigen–antibody interaction was studied by Ouchterlony double immunodiffusion method in the presence of glucose (Weir, 1977; Hankins *et al.*, 1979).

Peptide maps: The urea denatured lectin (5 mg) was suspended in 1 ml of ammonium bicarbonate pH 8·5 and kept at 37°C for 8 h after addition of TPCK trypsin (enzyme substrate ratio 1:50). The TPCK treated trypsin was added in two portions at 4 h intervals. At the end of the period the reaction was terminated with acetic acid and the material freeze dried. The tryptic digest was processed as described in literature for peptide maps (Rajagopal Rao *et al.*, 1973). Peptides were located on thin-layer chromatography plates using fluorescamine reagent (Udenfriend *et al.*, 1972). Arginine peptides were detected with phenanthroquinone reagent (Yamada and Itano, 1966).

Haemagglutination activity: The haemagglutinating activity and sugar inhibition studies were carried out using 2% trypsinised rabbit erythrocytes according to the procedure of Paulova *et al.* (1971). The unit of activity is defined as the lowest concentration of the lectin giving visible agglutination. The sugar inhibition assays were carried out as follows. To each 0·01–0·1 ml of sugar solutions in 0·9% saline containing 1–10 μmol sugar and 4 haemagglutinating units of either lectin in a total volume of 0·2 ml was added and incubated for a period of 1 h at 27°C. Later 0·2 ml of trypsinised rabbit erythrocytes or human O group erythrocytes were added and incubated for 2 h at 37°C. The lowest concentration of sugar which inhibits agglutination was taken as the inhibitory titre of the sugar. The inhibition experiments with various complex glycoproteins and glycopeptides were carried out as described in literature (Debray *et al.*, 1981, 1983).

Metal content: Metal analysis of the lectins after ashing was carried out by atomic absorption on a Varian techtron 1000 Spectrophotometer.

Gel electrophoresis: Polyacrylamide gel electrophoresis (7·5 % acrylamide) at different pH values was carried out according to Reisfeld *et al.* (1962), Davis (1964) and SDS gel electrophoresis was carried out as described (Weber and Osborn, 1969).

Ultracentrifugal studies: Velocity sedimentation experiments were carried out at 27°C in a Beckman Model E analytical ultracentrifuge (56,100 rpm) equipped with an RTIC unit. A standard 12 mm Kel F cell was used for all the experiments. The $S_{20,w}$ values were calculated using standard procedures (Schachman, 1959). The partial specific volume of the lectins was found to be 0·73 from the amino acid composition (Cohn and Edsall, 1943). For sedimentation experiments the protein concentration was 10 mg/ml.

Gel Filtration: The molecular weight (M_r) of the lectins was determined by gel filtration on Biogel P-200 (Andrews, 1970). Standard proteins such as bovine serum albumin (68,000), ovalbumin (45,000), pepsin (EC 3.4.23.1) (35,000), α-chymotrypsin (EC 3.4.21.1) (25,000) and lysozyme (14,700) were used for calibration. The subunit M_r of the lectins was determined on Sepharose 6B using 6 M guanidine hydrochloride (Mann and Fish, 1972). Standard proteins such as α-chymotrypsin (25,000), β-lactoglobulin (18,000), lysozyme (14,700) and cytochrome C (12,700) were used for calibration. Further experiments for subunit M_r determination were also carried out on Biogel P-30 using 1 % SDS and on Biogel P-100 using 2 M acetic acid (Baumann *et al.*, 1982; Sletten *et al.*, 1983). α-Chymotrypsin, β-lactoglobulin, lysozyme and cytochrome C were used as standard proteins for Biogel P-30. Bovine serum albumin and ovalbumin were additionally used on Biogel P-100 along with the proteins used on Biogel P-30.

pH Stability of lectins: This was determined by exposing lectins (2 mg/ml) for a period of 24 h at 25°C to the following buffers: (i) 0·2 M KCl–HCl buffer, pH 2·0, (ii) 0·2 M sodium acetate–acetic acid buffer, pH 4·0, (iii) 0·2 M sodium phosphate buffer, pH 6·0, (iv) 0·2 M Tris-HCl buffer, pH 8·0, and (v) 0·2 M sodium carbonate–bicarbonate buffer, pH 10·0. The activity of the lectins was tested after dialysis of the above samples against 0·9 % saline.

Temperature stability of lectins: A solution of either lectin (2 mg/ml) was prepared in Pi/NaCl pH 7·4 and distributed into several tubes. Each of these tubes was kept at 50°C, 60°C, 70°C, 80°C and 100°C for a period of 15 min and then cooled in ice, centrifuged if necessary and assayed for haemagglutinating activity.

Circular dichroism studies: Circular dichroism (CD) measurements were made at 27°C in a JASCO J-20 C automatic recording spectropolarimeter calibrated with d-10-Camphor Sulphonic Acid. Quartz cells of 1 cm were used for measurements in the region 350–200 nm. Slits were programmed to give a band width of 10 Å at each wave length. The data are expressed as mean residue ellipticities in degrees $cm^2 \, d \, mol^{-1}$, taking a mean residue weight of 110 for both lectins. The effect of binding sugars on CD spectra was measured 16 h after mixing with protein solutions. The CD spectra were analysed by the method of Provencher and Glockner (1981).

Results and discussion

Purification

The defatted seed powder 50 g, either from the field or lablab bean was stirred with 250 ml of 0·15 M NaCl for 16 h at 4°C. After centrifugation the clear supernatant was collected and the residue reextracted with 0·15 M NaCl for 2–3 h. The combined supernatants were subjected to ammonium sulphate fractionation at 0–4°C. Initially, $(NH_4)_2SO_4$ (24·3 g/100 ml) was added and the precipitate collected after an hour by centrifugation. Subsequently to the supernatant (370 ml) $(NH_4)_2SO_4$ (13·2 g/100 ml) was again added and after few hours the precipitate was collected by centrifugation. The second precipitate was dissolved and dialysed against 0·15 M NaCl (3 × 2 litres) at 4°C. Later the protein solution was centrifuged at 10,000 g and the clear supernatant collected. Because of the presence of oxidised polyphenols the 40–60 % fraction is coloured, but these coloured components are not adsorbed on the affinity column and are removed initially. 100 A_{280} units of the supernatant was applied to an affinity column of D-mannose linked Sepharose (2·5 × 21 cm) which was previously equilibrated with Pi/NaCl. The column was washed with the same buffer until the absorbance of the effluent was less than 0·05 at 280 nm and the bound lectin was eluted with 0·25 M glucose in Pi/NaCl (figure 1). The fractions having the haemagglutinating activity were pooled, dialysed extensively against water or 0·15 M NaCl depending on the experimental requirements. The sample dialysed against water was freeze dried. Usually 0·025 μg/ml of either lectin is required to agglutinate rabbit erythrocytes under
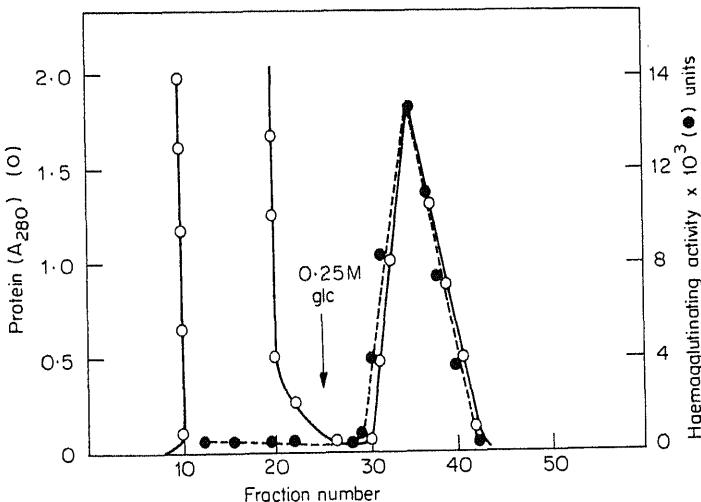

Figure 1. Affinity chromatography of *D. lablab* lectins on a column of D-mannose linked Sepharose 6B. 100 A_{280} units of the dialysed fraction after ammonium sulphate fractionation (40–60 %) obtained from 50 g of the defatted field bean meal was applied to a 2·5 × 21 cm column equilibrated with Pi/NaCl. Elution was with 0·25 M glucose. (O), A_{280}; (●), haemagglutinating activity (units).

standard conditions of assay. The freeze dried lectin is stable at 4°C for nearly 6 months without significant loss of activity. In the above purification procedure about 35% of the activity was recovered and about 3% of the soluble protein seemed to be the lectin (table 1).

The field and lablab bean lectins were not specifically held on Sephadex or concanavalin A (Con A) Sepharose columns and could not be purified by these methods. Nearly 75–85% of the field bean lectin purified by conventional methods (Narayana Rao *et al.*, 1976; Hariharan, 1979) was held on D-mannose linked Sepharose columns. The field and lablab bean lectins were stable upto 70°C at neutral pH. Exposure of the lectins to pH below 2 or above 8 at room temperature (27°C) results in the loss of their ability to agglutinate erythrocytes.

Physico-chemical properties

The purified lectins were homogeneous by polyacrylamide gel electrophoresis at pH 4·6 over a concentration range of 25–150 μg (figure 2A). Also at pH 3·1 and 9·2 only a single component was noticed on the gel. Polyacrylamide gel electrophoresis in the presence of SDS showed two closely migrating bands with apparent M_r of 15,000 and 12,000, the band of 15,000 MW staining more intensely than the band of 12,000 MW (figure 2B). The lectin preparations gave a single symmetrical peak with a $S_{20,w}$ value of 5·2 in sedimentation velocity experiments. Under the same conditions the total proteins of the field and lablab bean sedimented with at least two major components with $S_{20,w}$ values of 2 and 7, besides a fast moving minor component (figure 3). The M_r of the lectins by gel filtration on Biogel P-200 was found to be around 60,000. Gel filtration of the lectins on Biogel P-30 in 1% SDS indicated a single component with M_r of about 15,000. Gel filtration of the lectins on Sepharose 6B in 6 M guanidine hydrochloride also indicated a single component with a M_r of 15,000. Sedimentation experiments of the lectins in guanidine hydrochloride gave a $S_{20,w}$ value of nearly 1·0. Gel filtration of the lectins on Biogel P-100 in 2 M acetic acid indicated a major component with a M_r of 17,000 and a minor component with a M_r of more than 100,000. In the analytical

Table 1. Purification of either field bean or lablab bean lectin (the crude extract was obtained from 50 g of defatted field bean meal).

Step	Volume (ml)	Total proteins (mg)	Specific activity (units/mg*)	Total activity × 10⁵	Recovery (%)
Crude extract	345	8142	1086	88·4	100
(NH₄)₂SO₄ fractionation					
0–40% fraction	14	357	1288	4·6	5·2
40–60% fraction	21	1518	3570	54·2	61
60–80% fraction	12	306	620	1·9	2·1
Affinity					
chromatography	134	241	12739	30·7	34

* One haemagglutination unit is defined as the minimum concentration of protein required to cause agglutination.
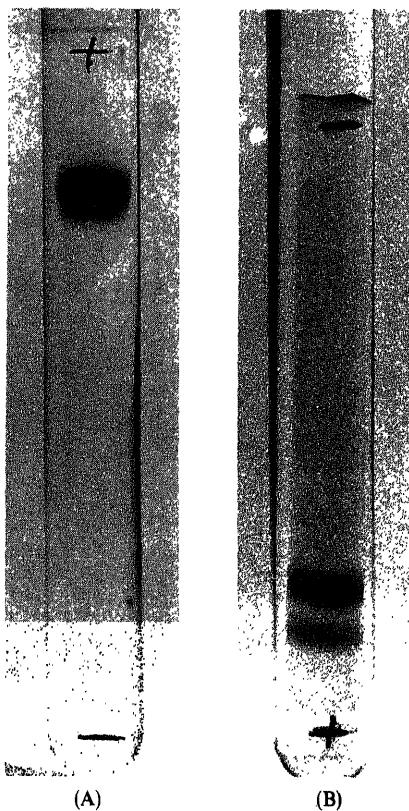
Figure 2. Polyacrylamide gel electrophoresis of purified field bean lectin. (A) 7·5 % acrylamide, pH 4·6, protein concentration 150 μg. (B) 10 % acrylamide in presence of SDS, pH 7·2, protein concentration 100 μg.

ultracentrifuge at acid pH (2 M acetic acid) the lectin showed two components, the major component with a $S_{20,w}$ value of $\sim 1\cdot0$. The minor component ($S_{20,w} = 11$) is probably the aggregated form of the lectin as many of the seed proteins are known to form aggregates at low pH values and conditions of high molarity of acids (Prakash and Narasinga Rao, 1984). The results suggest that the lectins consist of 4 similar subunits of M_r 15,000.

The lectins are glycoproteins containing mostly fucose, mannose and glucosamine. Small quantities of xylose could also be detected by gas liquid chromatography procedures. By analysis of different batches of lectins we found that the sugars are in the following range 2–5 mol of fucose, 5–8 mol of mannose and 1–2 mol of glucosamine per mol of the lectins.

The amino acid composition of the field and lablab bean lectins are very similar (table 2) with higher amounts of acidic amino acids and lack of methionine and half cystine in common with other glucose/mannose specific lectins (Allen *et al.*, 1972). Further the proteins have similar tryptic peptide maps (figure 4). The peptide map showed 8 spots on staining with fluorescamine and 3 of these spots gave a positive test

(A)   (B)
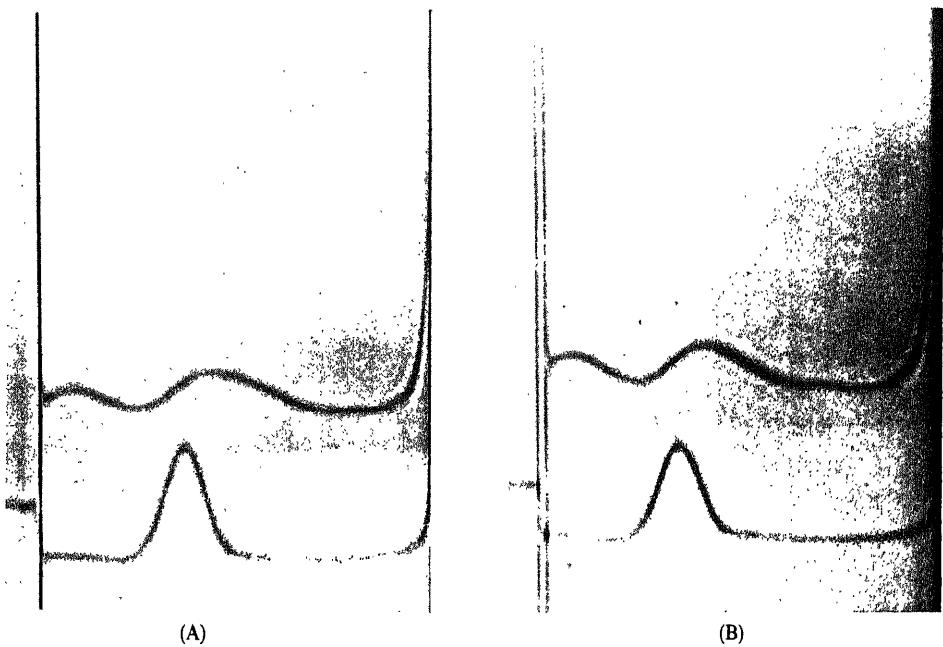
Figure 3. Sedimentation pattern of (A) field bean and (B) lablab bean lectins. The upper pattern is that of total proteins and the lower that of the affinity purified lectins respectively. Sedimentation is from left to right. Several photographs were taken at different time intervals (A) 53 min and (B) 55 min.
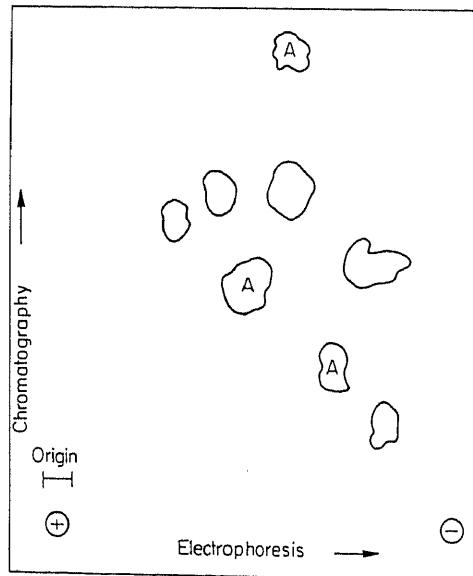

Figure 4. Tracing of peptide map of tryptic digest of field bean lectin. "A" denotes arginine peptides. The peptide map of lablab bean lectin was virtually identical with that of the field bean lectin.

Table 2. Amino acid composition of field bean and lablab bean lectins.

Amino acid	mol/mol protein	
	Field bean	Lablab bean
Asp	53	57
Thr	35	34
Ser	51	58
Glu	33	39
Pro	19	19
Gly	30	30
Ala	37	38
Val	29	31
Met	—	—
Cys	—	—
Ileu	34	36
Leu	37	37
Tyr	24	24
Phe	28	26
His	14	12
Lys	19	20
Arg	13	13
Trp*	10	10

* Tryptophan was determined spectrophotometrically by the method of Edelhoch (1967). No corrections have been made for loss of serine and threonine during acid hydrolysis. Aspartic acid and glutamic acid values include aspargine and glutamine respectively. Calculations are based on a M_r of 60,000 for both field bean and lablab bean lectins. The values have been rounded off to whole numbers. Methionine and half cystine could not be detected.

for arginine peptides when stained with phenanthroquinone reagent. From the amino acid composition the number of arginine and lysine residues per mol of protein are 13 and 19 and a tryptic peptide map should show 9 peptides if the subunits are identical. The experimental finding of 8 tryptic peptides is a good evidence for the presence of identical subunits. In both lectins amino terminal analysis indicated alanine as the only end group and carboxypeptidase treatment revealed that serine was the first amino acid released. From the subunit M_r, end group analysis and tryptic peptide maps it seems most likely that the lectins are oligomeric proteins made of 4 similar subunits. The lectins contain low amounts of bound metals. About 0·4 mol of manganese, 0·6 mol of magnesium and 0·15 mol of calcium are present per mol of protein in both the lectins. The lectins do not lose their haemagglutinating activity on dialysis against 0·1 M ethylenediamine tetraacetic acid (EDTA), however, the activity is lost on dialysis against 0·1 M acetic acid. The activity could not be restored by addition of external source of metal ions.

Immunological properties

In order to compare the field and lablab bean lectins with other glucose/mannose specific lectins described in literature, antibodies were raised for these two purified lectins in rabbits. By Ouchterlony double immunodiffusion technique it was found that the antiserum from the field bean lectin cross reacts with the lablab bean lectin or *vice versa*, suggesting the presence of similar antigenic sites in both lectins. There was no spur formation when the lectins were placed in adjacent wells. However, neither of the antisera cross react with other glucose/mannose specific lectins such as Con A, lentil or pea (figure 5).

Sugar specificity

The majority of glucose/mannose specific lectins reported in literature agglutinate human A, B, O erythrocytes as well as rabbit erythrocytes. The lectins from the field and lablab beans also agglutinate the A, B, O erythrocytes and rabbit erythrocytes (table 3). In order to establish the sugar binding specificity of these lectins, a number of sugars have been used to check the inhibition of haemagglutination. Of the various sugars tested the most effective were glucose, mannose, their α-derivatives, trehalose and 3-*o*-methyl glucose (table 4). These sugars probably bring about inhibition as a result of interaction with the carbohydrate binding sites of protein. Inhibition by β-D-methyl glucopyranoside was very low when compared to the α-derivatives. Substitution at the third position enhances the ability of the sugar to inhibit more strongly. The configuration around C-2 does not seem to be important for binding.
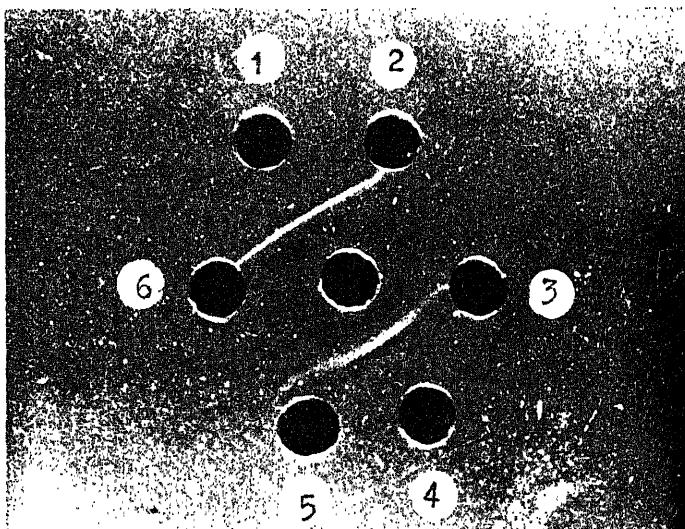

Figure 5. Immunodiffusion of the purified lectins. Central well has 20 μl of antiserum to the field bean lectin. Wells, 1 and 4, contained 20 μg of field and lablab bean lectins respectively. Wells, 2, 3, 5 and 6 contained 20 μg of Con A, lentil, pea, and *D. biflorus* lectins respectively.

Table 3. Agglutination of erythrocytes of various species by the field bean and lablab bean lectins. Experimental conditions are given under 'materials and methods'.

Origin of erythrocytes	Minimal haemagglutinating dose	
	Untrypsinised	Trypsinised
	μg/ml	μg/ml
Human A type	7·25	3·625
Human B type	14·5	7·25
Human O type	14·5	7·25
Human AB type	14·5	7·25
Rabbit	0·45	0·025
Fowl	0·45	0·05
Guinea pig	0·45	0·05
Sheep	a	a
Cow	a	a

a No agglutination at 1 mg lectin/ml.

Table 4. Inhibition of haemagglutination by various sugars.

Inhibitor	Minimal inhibitory concentration (mM)	
	Rabbit erythrocytes	Human O^+ erythrocytes
D-Glucose	5·0	2·5
D-Mannose	5·0	2·5
Trehalose	2·5	2·5
Methyl α-D-glucopyranoside	2·5	5·0
N-Acetyl glucosamine	10·0	10·0
Methyl α-D-mannopyranoside	5·0	7·5
3-o-Methyl glucose	2·5	a
Sepharose	7·5	a
Maltose	7·5	a

Other sugars tested, *i.e.*, L-fucose, D-fructose, D-galactose, L-rhamnose, and Raffinose were not inhibitory at concentrations of 0·1 M. The minimal inhibitory concentration is that required to inhibit 4 haemagglutination units of either lectin. One haemagglutination unit is equivalent to 0·08 μg of either lectin when tested with rabbit erythrocytes and 0·25 μg of either lectin when tested with human O^+ erythrocytes.
a Not inhibitory at concentrations of 0·1 M.

L-Fucose, D-galactose, D-fructose, D-arabinose, D-xylose, L-rhamnose, lactose, cellobiose, melibiose and raffinose were not inhibitory at concentrations of 0·1 M. Among a number of complex glycoproteins such as human serotransferrin, human

lactotransferrin, glycopeptides from human serotransferrin, glycopeptides from bovine lactotransferrin, ovomucoide, glycopeptides from ovomucoid, orosomucoid tested for inhibition of lectin activity using human O^+ erythrocytes it was found that the lectin activity was best inhibited by glycopeptides which have a number of N-acetyl glucosamine residues (Debray *et al.*, 1981, 1983) in the terminal non-reducing position (table 5).

Con A has the remarkable ability to precipitate polysaccharides such as glycogen, dextrans, mannans etc., in the presence of metal ions such as Ca^{2+} and Mn^{2+} (Goldstein and Hayes, 1978). In contrast to Con A, field bean and lablab bean lectins did not precipitate any of the polysaccharides.

CD studies

CD studies have been widely used for determining the secondary and tertiary structures of many lectins. These studies have also been extended to investigate the effect of specific saccharide binding on protein conformation in solution. We have carried out the CD studies for the field and lablab bean lectins in the far and near ultra-violet (UV) region and found that the lectins have similar far UV and near UV CD spectra (figure 6). The far UV spectra resembles that of other lectins reported in literature with about 60% β-sheet structure. However, the near UV CD spectra of both lectins are quite different from other glucose/mannose specific lectins in that the negative bands are seen in the 275–290 nm region. In this respect the lectins from the field and lablab bean resemble Sainfoin another glucose/mannose specific lectin (Young *et al.*, 1982).

Table 5. Inhibition of haemagglutination by different glycoproteins and glycopeptides*.

Glycoprotein or glycopeptide	Lectin used		
	Field bean or lablab bean	*Lens culinaris*	Con A
Human serotransferrin	0·033		
Human lactotransferrin	0·0082		
Glycopeptides from human serotransferrin	0·415		0·0515
Glycopeptides from human lactotransferrin	0·83	0·0015	
Glycopeptides from bovine lactotransferrin	1·66	0·207	0·0015
Ovomucoid	0·00205	0·033	0·0165
Glycopeptides from ovomucoid	0·025	0·415	0·103
Orosomucoid α-1-acid glycoprotein	0·033		

One haemagglutination unit of either lectin is 15·75 μg/ml. Minimal inhibitory concentration is that required to inhibit 4 haemagglutinating units of untrypsinised human O^+ erythrocytes. Concentrations of the glycoproteins and glycopeptides are in mM. The structures of the glycopeptides are described earlier (Debray *et al.*, 1983).
* These results were obtained in collaboration with Dr. H. Debray.
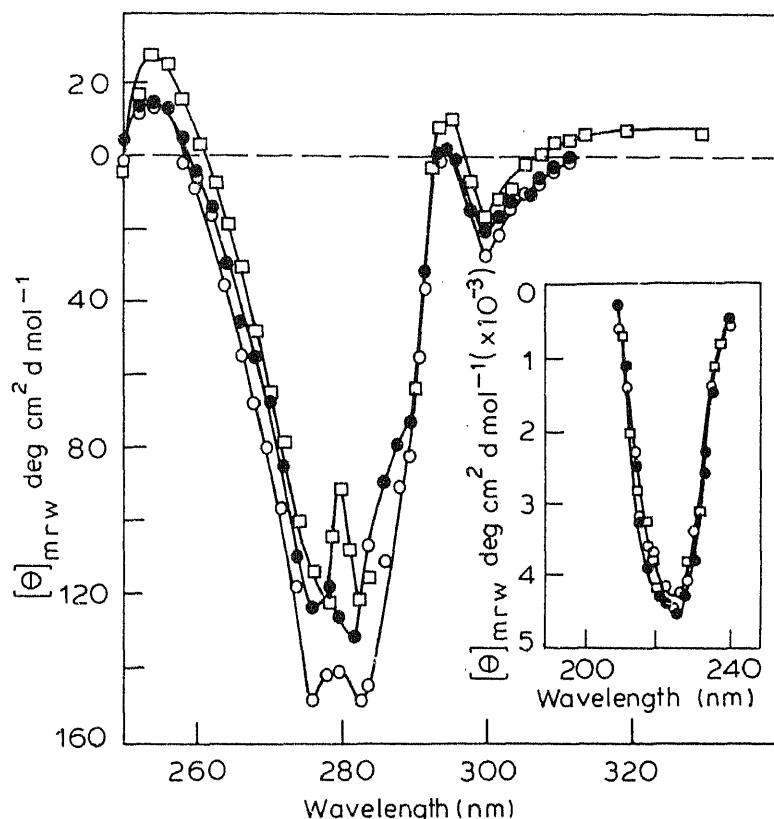
Figure 6. Effect of methyl α-D-glucoside on the CD spectra of the field bean lectin in the far and near UV regions. (O), Field bean lectin in 0·15 M NaCl; (●), lectin with 5 mM sugar; (□), lectin with 1 mM sugar.

Different concentrations (5, 1 and 0·5 mM) of the binding sugar such as methyl α-D-glucopyranoside had a marked effect on the near UV CD spectra of the lectins. There is a decrease in the magnitude of bands in the 250–310 nm region. The effect was more pronounced at lower concentrations of sugar. This shift in the CD spectra in the tyrosine and tryptophan bands in the presence of binding sugars suggests that probably these aromatic residues are involved in the sugar binding site of the lectins. Similar results have been obtained with Con A by CD experiments (Pflumm *et al.*, 1971). We have additional evidence by chemical modification experiments in the presence of sugars by group specific reagents that tyrosine and tryptophan residues are involved in carbohydrate binding (N. Siva Kumar and D. Rajagopal Rao, unpublished data).

Guran *et al.* (1983) have reported the purification of a lectin from field bean whose carbohydrate specificity seems unusual for a glucose/mannose specific lectin. They reported that agglutination of human 'O' erythrocytes was inhibited by N-acetyl-D-glucosamine, methyl α-D-mannopyranoside and ovomucoid but not by D-glucose, D-mannose or trehalose. The M_r was reported to be around 110,000 comprising of two

α-subunits (M_r—16,000) and two β-subunits (M_r—40,000). The amino acid composition of the lectin as well as the presence of zinc in the protein indicate that it does not resemble either of the lectins we have purified and described in the present report.

From the experimental data presented above such as dissociation of lectin and gel filtration, peptide maps, end group analysis, sedimentation analysis and *in vitro* translation studies (N. Siva Kumar, D. Rajagopal Rao and G. Padmanaban, unpublished data), it is clear that the field and lablab bean lectins are most likely homotetramers. The only data that is at variance with the above conclusion is the identification of two components by SDS gel electrophoresis. The possibility that these components arose by proteolytic action as in the case of other lectins like Con A and soyabean (Wang *et al.*, 1971; Lotan *et al.*, 1975) was also checked by using inhibitors such as phenylmethylsulphonyl fluoride but no significant differences could be found. Further the finding of a single N-terminal and C-terminal amino acid argues against such a proteolytic modification. The anamolous behaviour of the lectin in SDS gel electrophoresis may possibly be due to its glycoprotein nature. The general properties of the field bean and lablab bean lectins resemble those of other glucose/mannose specific lectins specially that of Sainfoin (Hapner and Robbins, 1979).

Dolichos biflorus (horse gram) has been classified botanically as belonging to a different genus and is treated as synonymous with *Macrotyloma uniflorum* (Verdcourt, 1970). It is not surprising that the lectins of the lablab and field bean are completely different in their physicochemical and biological properties from the very well studied *D. biflorus* lectin (Etzler, 1972).

Acknowledgements

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A new method of measurement of permeability of lipid membranes

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Abstract. A new method, based on potential measurements to obtain permeabilities and mobilities of ionic species passing through membranes, under the influence of concentration gradients is proposed. This method is applied to three different lipid membranes in aqueous KCl solutions. The values of permeabilities and mobilities obtained by this simple method is in good agreement with those reported in literature.

Keywords. Permeability; lipid membranes; flux measurement

Introduction

The mechanism of transfer of ionic species across biological membranes is of crucial importance in understanding the signal transmission in living cells. To this end, extensive studies of K^+, Na^+, Cl^- and other ion transport across natural and lipid membranes have been reported. The flux measurements of ions under different conditions such as pure concentration gradients, electric potential gradients and a combination of these two would enable one to calculate the permeabilities of the membranes to different ions under given conditions. These permeability values can then be used to (i) characterise the membranes, (ii) calculate the rest potentials of the membranes and (iii) understand the action potentials. Thus permeabilities play an important role in the study of membranes.

Methods that are available to determine permeability

(i) Radioactive sample of the ion to be determined (*eg.* ^{203}Hg in the case of mercury, ^{12}C in the case of Carbon) is injected into compartment one. The rate of appearance of radioactivity in the compartment two was measured by continuous removal of aliquots at fixed intervals of times and then estimating the concentration using liquid scintillation counter. The flux (J) is then calculated using the relation

$$J = \frac{[C]_2}{tA\,SA_1},\tag{1}$$

J = Flux in mol $m^{-2}\,S^{-1}$, C_2 = total amount of tracer entering the compartment two in the time interval 't' s. SA_1 = specific activity of tracer in compartment one. Knowing J, permeability (P) is calculated using the relation (Walter *et al.*, 1982; Lakshminarayanaiah, 1969),

$$P = \frac{J}{C}.\tag{2}$$

(ii) The initial potential across the membrane is measured. Then a potential equal to the measured potential is applied to the system in the opposite direction. The current (I) in the presence of this applied potential is measured and then the flux is calculated by using the relation,

$$I = ZFAJ. \tag{3}$$

Where Z = Charge of the species; F = Faraday; A = Area of the membrane; P is calculated from J as given in the previous method (Kotyk and Janacek, 1970; Cereijido and Rutunno, 1970).

(iii) A known potential (E) is applied to the system by using two identical electrodes. The resulting current I is measured. The resistance (E/I) of the membrane and hence the conductance, G, which is the reciprocal of the resistance, *i.e.* I/E, are calculated. The permeability is then obtained using the relation (Gutknecht and Walter, 1982; Hodgkin, 1951; Dilger *et al.*, 1979)

$$P = \frac{GRT}{CF^2Z^2}. \tag{4}$$

Methods

We propose a new method of calculating the permeability using potential measurements in this paper. The method consists of separating two solutions of the same salt at different concentrations by the membrane and measuring the potential of this cell with respect to time using electrodes reversible to one of the ions. The flux of the ions with respect to time decreases the concentration gradient as a result of which the potential across the cell changes with time. Since we do not apply any external electric field and employ electrodes reversible to ions that are getting transported we assume Nernst equation to be applicable to the measured potentials. We also assume the transport rate is limited by the membrane. The method of calculating the permeability is given below.

Let C_1^A and C_1^B be the concentrations of the salt in compartments A and B at time t_1. Let the potential difference at time t_2 be E_2 and the concentrations C_2^A and C_2^B. At time t_3 the potential difference be E_3 and concentrations C_3^A and C_3^B and so on. Then we have

$$E_1 = \frac{RT}{nF} \ln \frac{C_1^A}{C_1^B}, \tag{5}$$

$$E_2 = \frac{RT}{nF} \ln \frac{C_2^A}{C_2^B} = \frac{RT}{nF} \ln \frac{C_1^A - x_1}{C_1^B + x_1}. \tag{6}$$

Knowing C_1^A, C_1^B which are the initial concentrations in the two compartments and measuring E_1 and E_2, x_1 can be calculated.

$$n_i = \frac{x_i}{1000} \times V, \tag{7}$$

where 'V' is the volume in m^3 of the solution in each compartment;

$$\text{Flux} = \frac{n_i}{(t_2 - t_1)A}, \tag{8}$$

where A is the area of the membrane in m^2;

$$P = \frac{\text{Flux}}{C},$$ (9)

where P is the permeability in m/s;

$$\text{Mobility, } U = \frac{P}{(dE/dx)}$$ (10)

where dE/dx is the potential gradient across the membrane.

We can calculate the current corresponding to the diffusion flux by multiplying both sides of eq. (4) by 'F', the Faraday. This current would be the same as the current measured by eq. (6) (Kotyk and Janacek, 1970).

Results and discussion

Using the above procedure we calculated the permeabilities of KCl solutions across different lipid membranes formed on a filter paper substrate (Gupta *et al.*, 1977, Ram and Rizvi, 1982). The experimentally measured values of potentials with time for 3 lipid membranes along with the permeabilities and the average mobilities calculated are given in tables 1–3. The values of permeabilities and mobilities without any lipid, that is, with filter paper alone, are reported in table 4.

From the results given in tables 1–4, it can be seen that the measured potential values are higher in cases where lipids separate the two solutions compared to the potentials obtained in the case when simple filter paper separates the solutions. The higher values of potential in the presence of lipids indicate high resistance to the movement of ions.

Table 1. Potential, permeability and mobility responses with time for sorbitan tristearate.

Lipid:	Sorbitan tristearate
Solvent:	*n* Decane
Filter paper:	Whatman 40
Concentration ratio maintained:	100
Electrodes used:	Ag/AgCl
Salt solution:	KCl

Time (min)	E volts	P $m/s \times 10^8$	U $\dfrac{m/s}{v/m} \times 10^{10}$
65	0·1529	3·6	2·4
90	0·1522	1·9	1·2
120	0·1513	2·1	1·4
150	0·1500	3·2	2·1
180	0·1491	2·3	1·5
240	0·1478	1·8	1·2
270	0·1470	2·2	1·5
300	0·1466	1·2	0·8

Table 2. Potential, permeability and mobility responses with time for phosphatidyl choline.

Lipid: Phosphatidyl Choline

$$
\begin{array}{c}
\quad\quad\quad\quad O \\
\quad\quad\quad\quad \parallel \\
O \quad\quad CH_2OCR_1 \\
\parallel \quad\quad\; | \\
R_2CO-CH \quad\quad O \\
\quad\quad\quad | \quad\quad | \\
\quad\quad\quad CH_2OPO \quad X \\
\quad\quad\quad\quad\quad | \\
\quad\quad\quad\quad\quad OH
\end{array}
$$

Phosphatidyl—X

X = Choline; (β hydroxyethyl) trimethyl ammonium hydroxide

Solvent: *n* Decaane

Filter paper: Whatman 40

Concentration ratio maintained: 100

Electrodes used: Ag/AgCl

Time (min)	E volts	P $m/s \times 10^7$	U $\dfrac{m/s}{v/m} \times 10^9$
60	0·130	2·9	2·2
90	0·125	5·7	4·6
120	0·124	1·3	1·0
140	0·1205	7·3	6·0
180	0·120	0·56	0·47
210	0·118	3·2	2·7
240	0·114	7·0	6·1

Literature reports show resistances of the order of 10^4 cm^2 (it is the practice in membrane studies to report the resistances in ohm cm^2; *i.e.* resistance × area of the membrane) for filter paper barriers and 10^8 cm^2 for black lipid membranes (Jain, 1972).

The results indicate that the permeabilities decrease by two orders of magnitude in the case of sorbitan tristearate and one order of magnitude in the case of phosphatidyl choline. The results, thus not only show the qualitative trend of the changes of permeability but we could calculate the actual values of permeabilities and mobilities which compare well with the values reported in literature obtained by other methods (Mac Innes, 1961).

An estimate of the diffusion coefficient can be made from the value of permeabilities obtained in the case of filter paper. Thus we get

$$D = P \times d = 1.5 \times 10^{-6} \times 10^{-3} = 1.5 \times 10^{-9} \ m^2/s,$$

which is of the order we get in aqueous solutions, thereby showing that the diffusion in the filter paper is same as in the bulk solution.

Thus from the results obtained it can be seen that this simple method gives reasonably good values for permeabilities and mobilities.

Table 3. Potential, permeability and mobility responses with time for phosphatidyl ethanolamine.

Lipid: Phosphatidyl Ethanolamine

$$
\begin{array}{l}
\qquad\qquad\quad O \\
\qquad\qquad\quad \| \\
O\ \ CH_2OCR_1 \\
\| \ \ | \\
R_2COCH \quad\ O \\
\quad\ | \qquad\quad \uparrow \\
\quad\ CH_2OPO \quad X \\
\qquad\qquad | \\
\qquad\qquad OH
\end{array}
$$
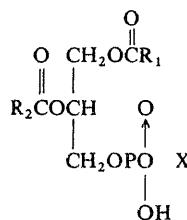
	Phosphatidyl--X
	X = CH$_3$CH NH$_2$ --
Solvent:	n Decane
Filter paper:	Whatman 40
Concentration ratio maintained:	75
Electrodes used:	Ag/AgCl

Time (min)	E volt	P $m/s \times 10^7$	U $\dfrac{m/s}{v/m} \times 10^9$
60	0·116	15·0	13·0
90	0·113	8·9	7·9
120	0·1115	4·8	4·3
150	0·1095	6·9	6·3
240	0·1035	8·6	8·3
270	0·1005	14·0	14·0
330	0·990	7·7	7·8

Table 4. Potential, permeability and mobility responses with time for filter paper.

Filter paper:	Whatman 40
Concentration ratio maintained:	100
Electrodes used:	Ag/AgCl

Time (min)	E volt	P $m/s \times 10^6$	U $\dfrac{m/s}{v/m} \times 10^8$
35	0·104	1·5	1·4
60	0·100	8·0	8·0
95	0·930	1·2	1·3
120	0·885	1·4	1·6
150	0·830	1·7	2·0
300	0·640	1·8	2·8
330	0·610	2·0	3·2
360	0·530	6·5	1·2

Acknowledgement

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Preformed messengers in *Microsporum canis* macroconidia

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Abstract. Macroconidia of *Microsporum canis*, when placed in a nutrient medium produce germ tubes within 4–6 h. Precursor incorporation studies showed that protein synthesis occurred prior to RNA synthesis. Sucrose density gradient analysis of wet and dry spore extracts revealed the presence of 16 % and 11 % polysomes respectively. The polysomal content increased to about 50 % within 15 min of germination. Synthesis of RNA occurred only after 2 h of germination. Pool equilibration of the radioactive precursors was not limiting to these measurements. Polyadenylated RNA was isolated from macroconidia and was found to comprise 2–2.5 % of the total RNA. The poly(A)$^+$ RNAs were heterodisperse and translatable in a wheat germ cell free translating system. It was concluded that macroconidia of *Microsporum canis* contain pre-formed mRNA which is translated early in germination.

Keywords. *Microsporum canis*; preformed mRNA; poly(A)$^+$ RNA; germination; polysomes; RNA/protein synthesis.

Introduction

The activation of a fungal spore from a metabolically quiescent state to a state of increased metabolism is a process that has been widely studied to understand cellular development and metabolic regulation in eukaryotic organisms. In most fungal spores, protein and RNA syntheses begin prior to germ tube emergence (Van Etten *et al.*, 1976). One of the foremost questions in fungal spore germination is whether it requires concomittant RNA synthesis or contains preformed mRNAs. Developmentally regulated changes in the syntheses of RNA and protein have been described in the life cycle of various eukaryotes, *viz.*, *Blastocladiella emersonii* (Leaver and Lovett, 1974), *Ustilago maydis* (Lin *et al.*, 1971), *Neurospora crassa* (Mirkes, 1974), *Rhizopus stolonifer* (Roheim *et al.*, 1974) and *Mucor racemosus* (Linz and Orlowski, 1982). In systems where germination proceeds in the absence of RNA synthesis, the translation of stable mRNA has been suggested as the source of proteins required for germination (Van Etten and Rawn, 1979; Johnson *et al.*, 1977; Linz and Orlowski, 1982).

 Microsporum canis is a pathogenic fungus which reproduces asexually by the formation of macroconidia (Vijaya Manohar *et al.*, 1977). *In vitro* studies have shown that protein synthetic activity in ungerminated conidia is low as compared to

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Abbreviations used: [^{14}C]-APH, [^{14}C]-Algal protein hydrolysate; TCA, trichloroacetic acid; UV, ultraviolet.

germinating conidia (Jayaram and Ramananda Rao, 1979) and it occurs prior to RNA synthesis. This report presents evidence for the presence of polysomes and poly(A)$^+$ RNA in macroconidia.

Materials and methods

Materials

[^{32}P]-Orthophosphoric acid (carrier-free) and [^{14}C]-algal protein hydrolysate ([^{14}C]-APH), sp. activity 40 mCi/atom of C, were purchased from Bhabha Atomic Research Centre, Bombay. [^{3}H]-uridine (sp. activity 12·7 Ci/mmol) was obtained from Amersham, England. [^{35}S]-Methionine was a gift from Mr. Subramaniam. PEI-cellulose sheets were gifts from Dr. Nagaraja, Biozentrum, Basel. Other chemicals used were of analytical grade.

Organism, macroconidia production and germination

M. canis strain HM 382 isolated from clinical lesions was maintained on Sabouraud's glucose agar slants. Macroconidia were obtained by the procedure described earlier (Jayaram and Ramananda Rao, 1979) and were germinated in Sabouraud's glucose medium (2% glucose, 1% peptone, 0·004% chloramphenicol and 0·004% cyclo-heximide) on a rotary shaker (150 rev/min) at 30°C.

Measurement of protein and RNA syntheses using radioactive precursors

For measurement of RNA synthesis, macroconidia were germinated in Sabouraud's medium containing [^{3}H]-uridine, 1 μCi/ml or [^{32}P]-orthophosphoric acid, 10 μCi/ml. Protein synthesis was monitored by using [^{14}C]-APH, 1·6 μCi/ml. 1 ml samples of the culture were withdrawn at different time points and placed in 1 ml of cold 20% trichloroacetic acid (TCA). After 1 h in ice, the samples were filtered through glass fibre filters, washed twice with 10 ml of 10% TCA followed by ethanol : ether (1 : 1) wash. The filters were dried and radioactivity counted as described earlier (Jayaram and Ramananda Rao, 1979).

Measurement of free intracellular nucleotide pools

Spores germinated in medium containing [^{32}P]-orthophosphoric acid for different time periods were collected on membrane filters and washed rapidly with water. Free nucleotide pools were extracted by the procedure described by Linz and Orlowski (1982). The nucleotides were separated by two dimensional PEI cellulose thin layer chromatography (Randerath and Randerath, 1967).

Density gradient analysis of ribosomes

Macroconidia (1–2 g) were harvested and washed with cold TMK buffer (50 mM Tris-HCl, pH 7·6, 10 mM magnesium acetate, 80 mM potassium chloride). The spores were disrupted by freezing and thawing with liquid nitrogen followed by grinding with glass powder. The extract in TMK buffer was centrifuged at 3,000 g for 5 min to pellet the

cell debris and unbroken spores. The supernatant was centrifuged again at 17,000 *g* for 15 min. The post-mitochondrial supernatant thus obtained was fractionated for ribosomes by centrifugation in a 10–40 % (w/w) linear sucrose density gradient in TMK buffer as described by Orlowski and Sypherd (1978). The gradients were centrifuged in a Beckman SW50 rotor at 35,000 rpm for 60 min at 4°C. The tubes were punctured and the fractions were passed through a scanner to measure absorbancy at 254 nm using Pharmacia dual path monitor or ultra-violet (UV) 2 equipped with a two-channel recorder REC-482. All steps were carried out at 4°C. Autoclaved glassware and solutions were used throughout.

Extraction, separation of RNAs and in vitro translation

RNA was extracted using saturated phenol:chloroform mixture and precipitated with ethanol (Perry *et al.*, 1972). It was fractionated on oligo(dT)cellulose (Aviv and Leder, 1972). Both total RNA and poly(A)$^+$ RNA were separated on 2–15% polyacrylamide gels having a gradient of 6–7 M urea in TBE buffer (89 mM Tris, 89 mM boric acid, 2·5 mM EDTA, 0·2% sodium dodecyl sulphate, pH 8·3). Electrophoresis was carried out in TBE buffer for 7 h at 100 V. The gels were stained using an aqueous solution of ethidium bromide (5 µg/ml) for 30–60 min. They were destained in distilled water and photographed in UV light. The poly(A)$^+$ RNAs were translated *in vitro* in a wheat germ system. The procedure was essentially that described by Erickson and Blobel (1983).

Results

RNA and protein synthesis during germination

Incorporation of labelled [^{3}H]-uridine into RNA is shown in figure 1. No significant incorporation occurred during the first 60 min after placing the spores in germination medium. However, the incorporation was significant after 80 min and continued through 5 h. In contrast protein synthesis occurred right from 10 min. The functional activity of polysomes observed in the early stages of germination was also examined. Macroconidia were grown in the presence of [^{14}C]-APH for 30 min and the extract was fractionated on sucrose density gradient. The fractions were monitored both for 254 nm absorbancy and for counts in TCA-precipitable material (figure 2). The results show that the polyribosomes found in germinating macroconidia have the capacity to become functionally active and bring about *in vivo* protein synthesis even during the first 30 min of germination. To rule out the possibility that the low incorporation was not due to limitation in uridine uptake, studies were carried out with [^{32}P]-orthophosphoric acid. As observed with [^{3}H]-uridine, [^{32}P]-incorporation was seen only after 90 min of germination (figure 3). The intracellular nucleotide pool was extracted from macroconidia germinating in [^{32}P]-labelled medium. The pools at different time points were separated on PEI-cellulose sheets for mono-, di-, and trinucleotides (figure 4).

Ribosome analysis in macroconidia

The level of polysomes present in ungerminated macroconidia of *M. canis* was determined (table 1). Mirkes (1974) had demonstrated in *N. crassa* that the water-
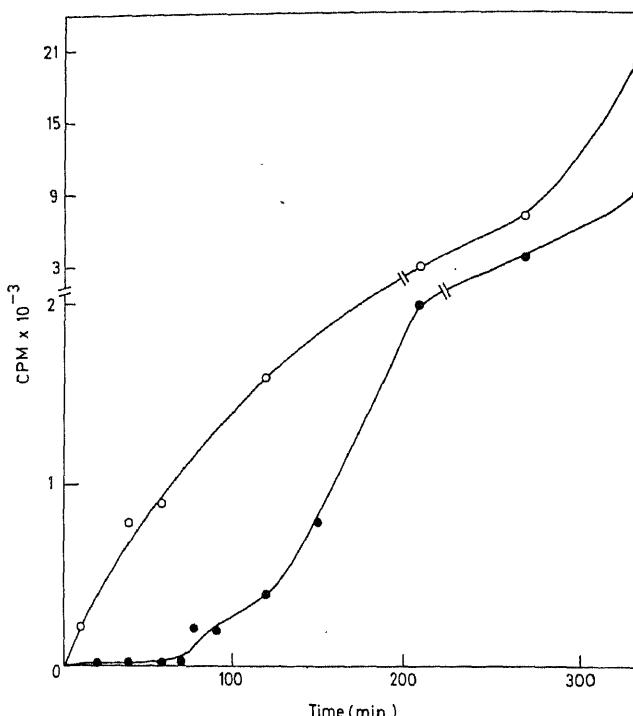
Figure 1. Incorporation of labelled precursors into TCA-insoluble material in germinating macroconidia of *M. canis*. (O), [^{14}C]-APH; (●), [^{3}H]-uridine.

harvested conidia are apparently activated and the polyribosome content was increased. In our studies we used heptane which had no adverse effect on the viability of macroconidia. The percentage of polysomes in wet and dry spore preparations were 16 and 11, respectively. Its content increased by over 4-fold to nearly 50 % within 15 min and remained at the level through 4 h of germination (table 1). To confirm the presence of polysomes, samples were treated with RNase. They were converted to monosomes while treatment with DNase had no such effect.

Analysis of RNA from macroconidia

Total RNA was extracted from macroconidia and analysed on 2–15 % polyacrylamide–urea gel. The gel pattern (figure 5A) showed the presence of all species of RNAs, *viz.*, ribosomal RNA comprising 25S, 18S, 5·8S and 5S, 4S transfer RNA, and heterogeneous mRNA. The total RNA was fractionated on oligo(dT)-cellulose column. The poly(A)$^{+}$ RNA constituted 2–2·5 % of the total RNA. On urea polyacrylamide gels it showed a heterodisperse pattern ranging in size from 6S–20S (figure 5B, lane 2). It was found to be translatable in the wheat germ cell-free translating system (figure 6).
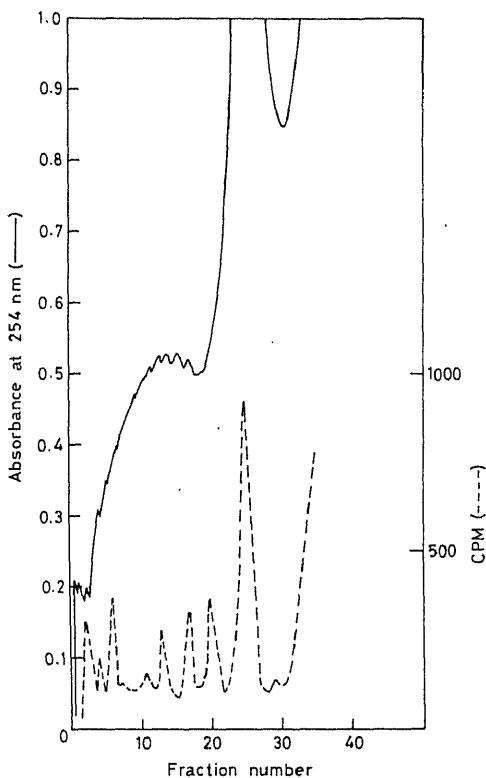
Figure 2. Functional activity of polysomes in germinating macroconidia. Macroconidia were germinated in medium containing 1·5 μCi/ml of [^{14}C]-APH for 30 min.

Discussion

During germination of *M. canis* macroconidia, protein synthesis can be detected within the first 30 min of germination. This is in contrast to the germination of spores in *Dictyostelium discoideum* and *U. maydis* where no measurable protein synthesis was observed upto 1 h and 6 h into germination, respectively (Yagura and Iwabuchi, 1976; Tripathi and Gottlieb, 1974). In fungal spores such as *Glomus caledonius* (Beilby and Kidby, 1982), *Fusarum solani* (Cochrane *et al.*, 1971), *R. stolonifer* (Van Etten *et al.*, 1974) an early and simultaneous RNA and protein syntheses were reported.

The polyribosome content in *M. canis* spores was largely unaffected when harvested with water. In contrast in *N. crassa*, dry spores contained only 3 % of polyribosomes whereas wet spores showed a 10-fold increase in the level of polysomes (Mirkes, 1974). However, the question about the activation of *N. crassa* spores by mere wetting is still open (Grange and Turian, 1981; Bonnen and Brambl, 1983). Brambl (1975) has shown that the content of polysomes in *Botryodiplodia theobromae* is same both in wet and dry spores. Similar observations have been reported by Linz and Orlowski (1982) in *M. racemosus*.
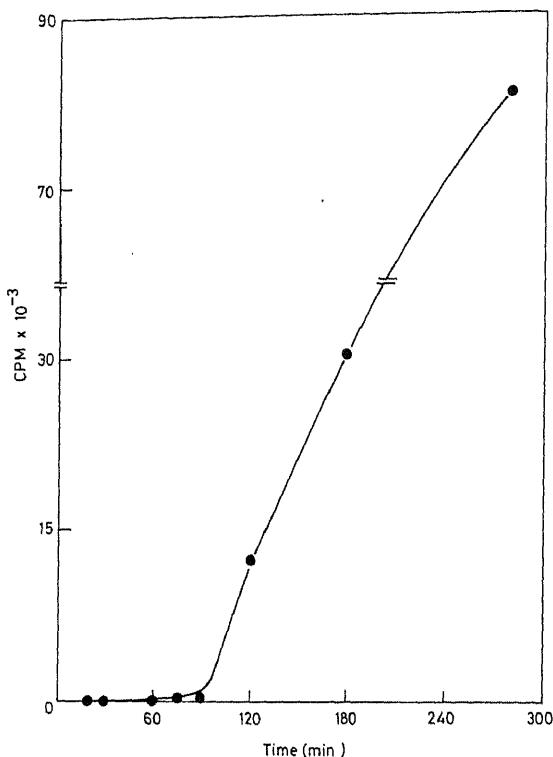
Figure 3. Incorporation of [^{32}P]-orthophosphoric acid into TCA-insoluble material during germination of macroconidia.

The kinetics of incorporation of [^{3}H]-uridine, [^{14}C]-APH, [^{32}P]-orthophosphoric acid do suggest that protein synthesis begins immediately upon placing macroconidia in the germination medium and continues for a period during which no detectable RNA synthesis occurs. The results obtained clearly bring out that macroconidia prior to germination do contain mRNAs which become functional rapidly upon initiation of germination. In *M. canis* macroconidia 2–2·5 % of total RNA is in polyadenylated form. Using wheat germ *in vitro* translating system, this poly(A)$^+$ RNA was found to be translatable.

Since the original proposal by Spirin (1969) that eukaryotic mRNAs exist as protein-bound complexes, a large number of reports in the literature indicate that mRNAs complexed with proteins (mRNPs) are of universal occurrence (Preobrazhensky and Spirin, 1978; Scherrer, 1980). The exact function of the proteins present in mRNP complexes is not known. Zoospores of *B. emersonii* contain mRNA which is not translated until the spores germinate (Lovett, 1975). It has been shown in *M. canis* macroconidia, that the mRNAs are present as mRNA-protein complexes which are translationally inactive. On deproteinization, the mRNAs become translationally active (Banumathi and Ramananda Rao, 1985). The translational activity of these mRNAs is probably regulated through selective recognition, resulting in their activation or repression.
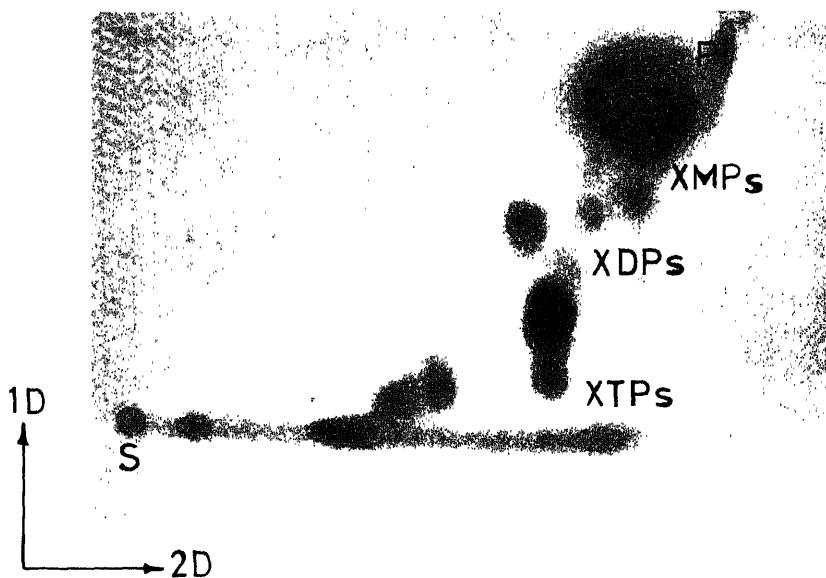
Figure 4. Labelled nucleoside phosphate pool at 10 min of germination. S is the point of application of sample. Pi is inorganic phosphate. XMPs, XDPs and XTPs are mono-, di- and trinucleotides.

Table 1. Polysome levels in macroconidia of *M. canis* during germination.

Germination time (min)	Ribosomes sedimenting as polysomes (%)
0 (dry)	11·3
0 (wet)	16·4
15	53·6
30	48·3
60	52·7
120	51·7
240	50·9

The post-mitochondrial supernatant from macroconidia germinated for different time periods was fractionated as given in 'materials and methods'. The areas under the monosome and polysome peaks were cut out and weighed. The level of polysomes is expressed as percentage of ribosomes sedimenting as polysomes.
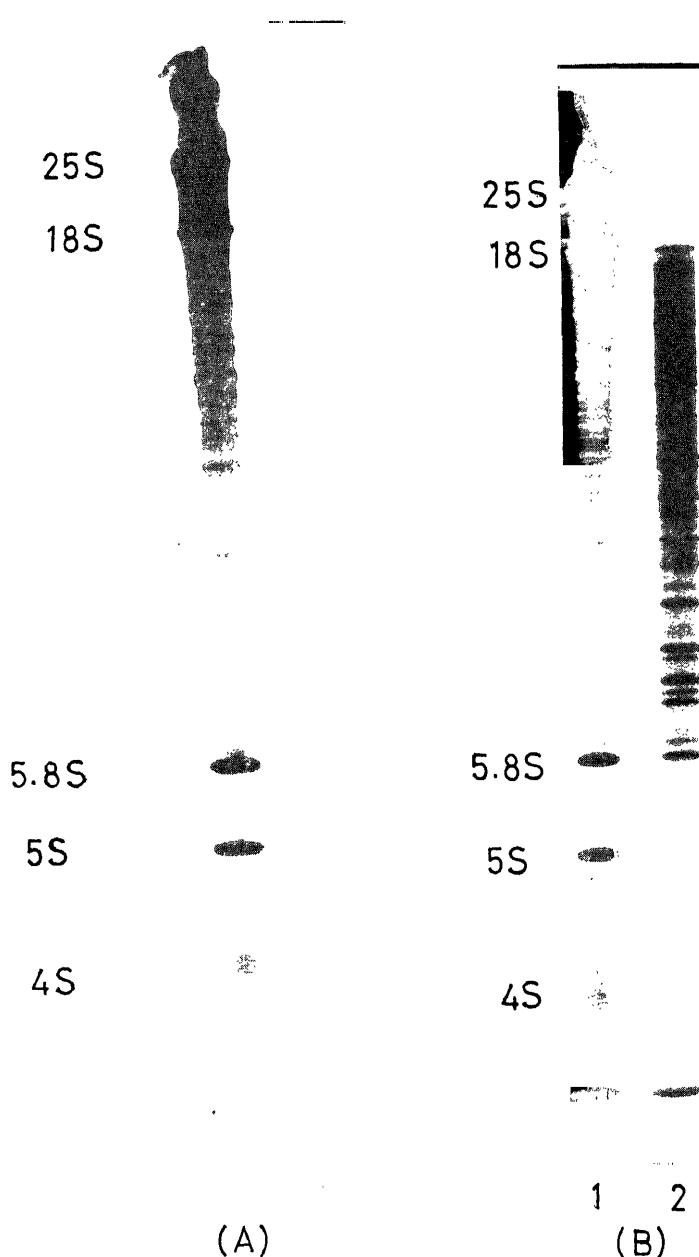
Figure 5. Urea-polyacrylamide gel electrophoresis of RNA. Samples 0·5–1·0 absorbance were electrophoresed. **A.** Total RNA from macroconidia. **B.** Polysomal RNA from *M. canis* as markers (lane 1), poly(A)$^+$ RNA (lane 2).
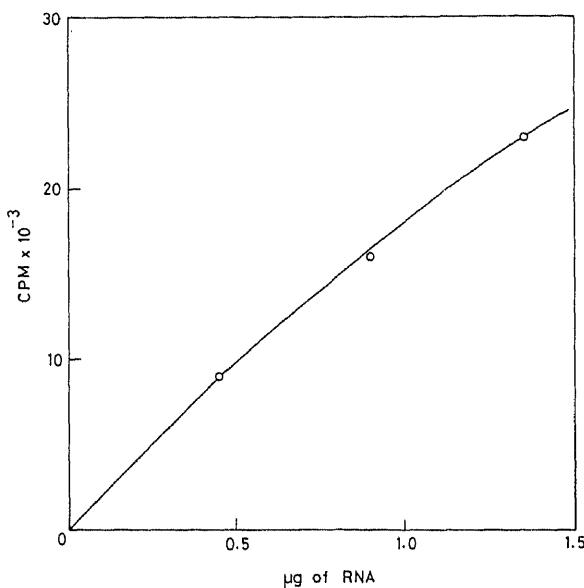
Figure 6. Translational activity of poly(A)⁺ RNA isolated from *M. canis* macroconidia. The RNAs were translated in a wheat germ cell free translating system. [³⁵S]-Methionine was used as the labelled amino acid.

Acknowledgement

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In vitro tests for screening of immuno-modulating mycobacterial strains in leprosy

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Abstract. There is an urgent need for the development of an *in vitro* assay for the initial screening of a large number of organisms from which potential candidates as vaccines can be identified. Our previous studies have demonstrated a crucial defect in the lepromatous macrophage. In this study by monitoring this defective macrophage response we have screened various mycobacteria for their ability to reverse the alterations induced by *Mycobacterium leprae*. Among the limited Mycobacteria tested *Mycobacterium vaccae* appears to be the most promising as an immunomodulator. Our results also indicate the need for caution in using the mouse model for this purpose.

Keywords. Leprosy; immuno-modulating strains; *in vitro* screening.

Introduction

The production of vaccine is a subject of high priority in the field of leprosy at present especially due to the emergence of drug resistant strains of *Mycobacterium leprae*. The organisms used for the production and testing of a vaccine at present consist of *M. leprae* (WHO 1983). *M. leprae* and BCG (Convit *et al.*, 1982), ICRC (Deo *et al.*, 1981), Mycobacterium W (Chaudhury *et al.*, 1983) and *M.vaccae* (Stanford *et al.*, 1981).

The absence of a quick, inexpensive method for testing potential vaccine candidates, has severely restricted the screening of organisms as suitable vaccine candidates. There seems to be a pressing need for the development of a quick *in vitro* assay system for the initial screening of a large number of organisms from which the most promising ones could be subjected to the much more expensive field trials.

Our previous studies have demonstrated a crucial defect in the response of the macrophage from lepromatous patients to *M. leprae* (Birdi *et al.*, 1983, 1984). The monitoring of this defective macrophage response might therefore prove to be a specific assay system.

The present study is an attempt to develop an *in vitro* assay using macrophages from lepromatous patients for screening of potential immuno-modulating mycobacteria, by checking the capacity of these mycobacteria to reverse the immune depression induced by *M. leprae*.

Abbreviations used: BI + ve, Bacteriologically positive; BI − ve, bacteriologically negative; MEM, minimal essential medium; EA rosetting, erythrocyte rosetting; SRBC, sheep red blood cells; MAIS, *Mycobacterium avium intracellulare scrofulaceum*.

Materials and methods

Choice of patients

Leprosy patients were classified according to the Ridley and Jopling (1966) classification. Lepromatous patients were further subdivided into bacteriologically positive (BI + ve) *i.e.* those who demonstrated acid fast bacilli in skin smears and bacteriologically negative (BI − ve) *i.e.* those who did not demonstrate acid fast bacilli in skin smears.

Macrophages

Human: Mononuclear cells were isolated from heparinized peripheral blood by sedimentation in 6 % Dextran, and freed from most of the lymphocytes by adherence to glass. The macrophages thus obtained were maintained for 7 days in minimal essential medium (MEM) containing 40 % human AB serum. The culture medium was changed after 24 h and subsequently after every 48 h. After 7 days in culture 95 % of the cells stained positive for non-specific esterase.

Mouse: The cells were obtained by irrigating the peritoneal cavity with MEM containing 20 % human AB serum. 0·7 ml of the cell suspension was dispensed into each leighton tube containing a coverslip. The culture was incubated at 37°C in 5 % CO_2 atmosphere. The cultures were maintained for 7 days during which time the medium was changed after the first 24 h, and subsequently after every 48 h.

Source of M. leprae

Biopsies of nodules from untreated lepromatous patients were homogenised and then trypsinized. The *M. leprae* obtained after differential centrifugation was washed with saline, stored at 4°C and used within a week.

F_c mediated erythrocyte rosetting

The erythrocyte rosetting (EA rosetting) was done 72 h after a 24 h infection of the macrophage cultures with 5×10^6 *M. leprae*. Sheep erythrocytes (SRBC) in a 2 % suspension in MEM were coated with an equal volume of goat anti SRBC antibody. A suspension of 1 % coated SRBC was overlayed on the macrophage culture and incubated at 37°C for 30 min. The monolayers were washed free of unbound SRBC, fixed in 2·5 % gluteraldehyde and stained with Ziehl–Neelsen acid fast stain. The percentage of cells with 2 or more SRBC attached was determined, after counting a total of 200 macrophages.

Antigen specific macrophage–lymphocyte physical interaction

Human: Mononuclear cells from peripheral blood of lepromatous (BI − ve) patients was isolated on a Ficoll–Triosil gradient.

The cells so obtained consisted of 80–90 % lymphocytes and 10–20 % macrophages. The cells were then resuspended in MEM containing 20 % human AB serum at a concentration of 4×10^6 cells/ml, and distributed into Leighton tubes containing

coverslips. The higher cell number was to allow for the varying numbers of monocytes finally obtained. After the appropriate mycobacterium (3×10^6 bacilli/tube) alongwith *M. leprae* (3×10^6/tube) was added the cells were incubated at 37°C for 18 h. The mycobacteria used were ICRC, BCG, *M. delhi*, *M. vaccae*, *M. avium*, *M. intracellulare* and *M. scrofulaceum*. The non-rosetted lymphocytes were washed off and the cells were fixed in 2·5% glutaraldehyde and stained with Ziehl–Neelsen acid fast stain. The percentage of macrophages with two or more lymphocytes adhering to it was calculated after counting a total of 200 macrophages.

Mouse: The mouse spleen was teased gently and the cells layered over Ficoll–Triosil. The cells were then washed and resuspended in 20% human AB serum in MEM at a concentration of 1×10^6 cells/ml. 0·7 ml was distributed into each Leighton tube. The appropriate antigen was added and the culture incubated for 18 h at 37°C in 5% CO_2 atmosphere. The termination and the staining procedures were similar to the human cell system.

In vivo mouse studies

The mice were infected subcutaneously with viable *M. vaccae* (1×10^6/mouse). At intervals of 6 months and 18 months the peritoneal macrophages were harvested and checked for EA rosetting. Simultaneously the spleen was collected for interaction.

Preparation of macrophage lysate

Macrophages were cultivated *in vitro* from the blood of BI − ve lepromatous leprosy patients. After 5 days in culture the macrophages were exposed to the following mycobacteria: *M. vaccae*, *M. intracellulare*, *M. scrofulaceum* and *M. avium*. After 48 h of exposure to the bacilli the macrophages were washed in saline. The cells were scraped off the glass with a rubber policeman and suspended in a known volume of saline and the cell count was determined. The cells were exposed to 6 cycles of freezing and thawing. The intracellular material thus released is referred to as the lysate. The lysate thus obtained was passed through a sterile millipore filter ($0·22 \mu$), to remove cell debris. Lysate was stored frozen till use. The lysate prepared in a similar manner from macrophages of normals and tuberculoid patient infected with *M. leprae* were termed as N-lysate and T-lysate respectively. The lysate of BI + ve lepromatous patients was termed as L–lysate.

Lymphocyte proliferation assay

Mononuclear cells from healthy individuals were separated over Ficoll–Triosil gradient. Cell count was taken and also checked for viability using trypan blue. The cell suspension was adjusted to 1×10^6 cells/ml in culture medium (MEM + 20% AB serum). Aliquots of 100 μl were distributed into each well of a microtitre plate. Fifty μl of antigen was distributed into appropriate wells. *M. leprae* was used at a dose of 3×10^6 bacilli/ml. Lysate prepared from 2×10^5 cells in 50 μl volume was also added to appropriate culture wells. Cultures were harvested on the 6th day. Each culture combination was set up in triplicate. Eighteen hours prior to harvesting the cultures 0·5 μCi of [^{3}H]-thymidine (specific activity 9·8 curies/mM) was added to each well.

Cells were harvested, processed and total thymidine incorporation was determined using the liquid scintillation counting system.

Results

Production of the suppressor factor

Our earlier studies have demonstrated that L-lysate was capable of inhibiting a normal lymphocyte proliferation to *M. leprae* antigen. However no suppressive activity was observed with N-lysate and T-lysate. These results suggest that an active interaction between lepromatous macrophages and *M. leprae* is required for production of the suppressor factor (Salgame *et al.*, 1984).

In this study other mycobacteria were tested for their ability to induce the production of the suppressor factor in lepromatous macrophages. Lysate prepared from macrophages infected with *M. vaccae*, *M. scrofulaceum* and *M. kansasii*, did not show any inhibitory activity (figure 1). Out of the 3 experiments performed with *M. intracellulare* and *M. avium* in two experiments no suppressive activity was observed. However, in one set of cultures some inhibitory activity was seen (figure 1).

Mycobacterial induced lymphocyte proliferation in the presence of L-lysate

M. leprae induced normal mononuclear cell proliferation was significantly reduced in the presence of L-lysate. Proliferation to mycobacterial antigens, *M. scrofulaceum*,
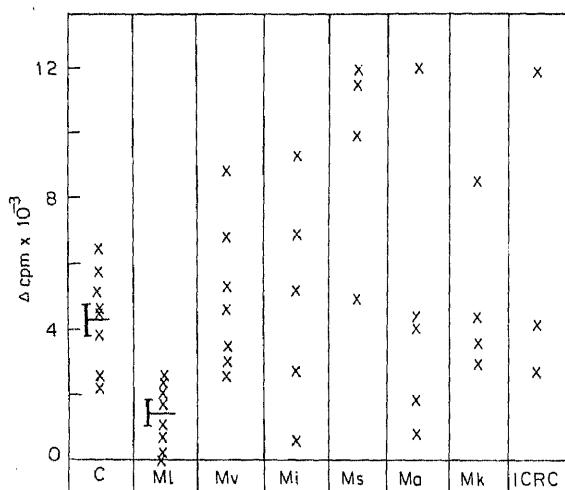
Figure 1. Effect of mycobacteria other than *M. leprae* on production of suppressor factor(s) from lepromatous bacillary negative macrophages. Each cross represents the normal mononuclear proliferation to *M. leprae* expressed as average counts per min of triplicate cultures of the same experiment. C, Lysate of macrophages not exposed to any bacilli. ML, Lysate of macrophages exposed to *M. leprae*. Lysate prepared in the presence of other mycobacteria. M.v, *M. vaccae*. M.i, *M. intracellulare*. M.s, *M. scrofulaceum*. M.a, *M. avium*. M.k, *M. kansasii* and ICRC.

Table 1. Lymphocyte proliferation of normal mononuclear cells to *M. leprae* and other mycobacteria in the presence of L-lysate as measured by [^{3}H]-thymidine incorporation.

Exp. No.				c.p.m.			
	1	2	3	4	5	6	7
Control	1608	859	395	3210	380	3488	289
+ *M. leprae*	3969	2328	507	6409	1825	15223	1244
+ *M. leprae* + L-ly	2313	1008	363	3200	1010	3059	525
+ *M. vaccae*	2073	2880		6671	2214	5436	
+ *M. vaccae* + L-ly.	2909*	2296*		6203*	2369*	5211*	
+ *M. scrofulaceum*	7137	8009		8887	16764		
+ *M. scrofulaceum* + L-ly.	3783	3813		2563	4438		
+ *M. intracellulare*	9217	9660		5873	6063	5749	
+ *M. intracellulare* + L-ly.	3620	2394		4385	4951	3904	
+ *M. avium*	6211	6419	4625	14752	2136		
+ *M. avium* + L-ly.	3096	1440	2306	4210	1832		
+ BCG	10956		1468			37807	9400
+ BCG + L-ly.	13615*		1220*			33631*	7535*
+ ICRC	13921			4546	577	33599	857
+ ICRC + L-ly.	3722			5336*	1586*	25696	912*
+ *M. delhi*	2894				1557	19643	
+ *M. delhi* + L-ly.	6258*				2370*	18208*	

* No significant suppression.

M. avium and *M. intracellulare* was also blocked by L-lysate. However, blastogenesis induced by *M. vaccae* and BCG was not inhibited by L-lysate. Out of 5 experiments with ICRC only in two cases stimulation was significantly lowered by L-lysate (table 1).

Lepromatous macrophage F$_c$ receptor expression

In an earlier paper (Birdi *et al.*, 1983) we have already described the effect of *M. leprae* infection of lepromatous (BI — ve) macrophage on EA rosetting. The percentage of EA rosetting was shown to be decreased in macrophage cultures infected *in vitro* with *M. leprae* as compared to the control monolayers of the same patient. From figure 2 it is evident that when the negative percentage difference was calculated between control cultures and *M. leprae* infected cultures a negative difference was obtained indicating a decreased 'EA' rosetting in the presence of *M. leprae*. Next the effect of the addition of a second mycobacterium was monitored. The mycobacteria used were those belonging to the *Mycobacterium avium intracellulare scrofulaceum* (MAIS) complex and the potential vaccine candidates enumerated to date. It appeared that *M. vaccae* was the only one able to totally abolish the effect of *M. leprae* and reverse the decrease in EA rosetting.

Macrophage–lymphocyte interaction in lepromatous patients

In the absence of any antigen in the system a baseline of less than 10 % interaction was obtained. In response to *M. leprae* macrophage–lymphocyte interaction in lepromatous patients was also below 10%, as reported earlier (Birdi *et al.*, 1984). Among the other mycobacteria tested, *M. vaccae* appeared to consistently enhance the interaction as evident from data presented in figure 3, while with the ICRC strains the results were
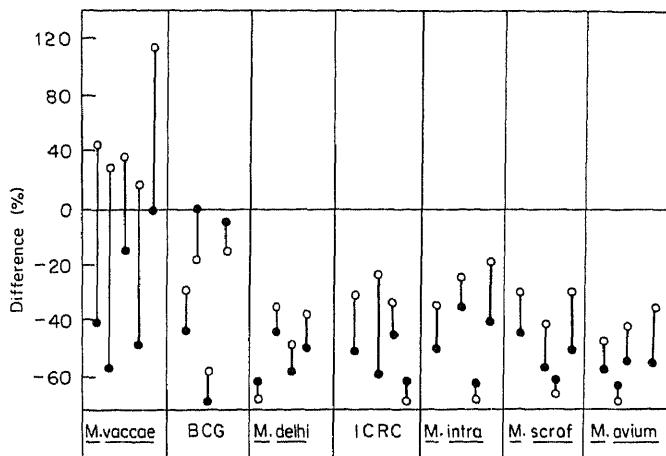
Figure 2. The effect of other mycobacteria on the decrease in EA rosetting in *M. leprae* infected lepromatous macrophages. The percentage difference was calculated taking the uninfected cultures as the baseline, *M. scrof.*, *M. scrofulaceum. M. intra.*, *M. intracellulare.* (●), M_i *leprae.* (O), *M. leprae* + Mycobacterium.
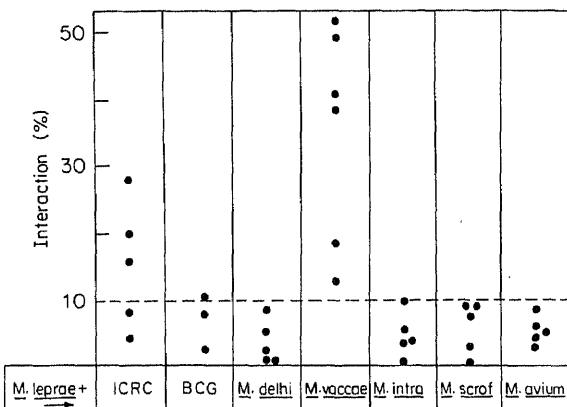
Figure 3. Macrophage–lymphocyte interaction in lepromatous patients in the presence of *M. leprae* (3×10^6) and the appropriate mycobacterium (3×10^6).

variable. Mycobacteria from the MAIS complex were not capable of inducing any increase in the interaction.

From these systems it was evident that among the mycobacteria tested only *M. vaccae* was consistently effective. Therefore, further experiments were done only with *M. vaccae*.

F_c receptor expression by peritoneal macrophages from Swiss white and C57B1/6 mice

In Swiss white mice there was a decrease in EA rosetting values in cultures infected *in vitro* with *M. leprae* when compared with control cultures. This was not observed in
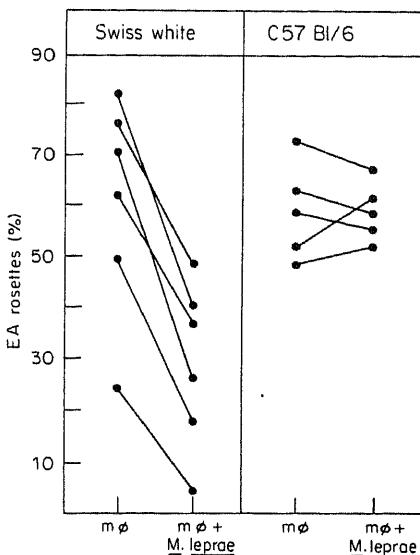
Figure 4. EA rosetting of peritoneal macrophages from Swiss white mice and C57 B1/6 mice after *in vitro* infection with viable *M. leprae*.

infected cultures of C57B1/6 (figure 4). Thus, peritoneal macrophages from Swiss white and C57B1/6 mice behave similarly to macrophages from lepromatous and tuberculoid patients respectively (Birdi *et al.*, 1983).

Since the *in vitro* tests reported may be used only as indicator systems for effective immunotherapy but not immunoprophylaxis, an attempt was made to study the memory component using the mouse as a model. However our attempts failed since the Swiss white mouse responds to *M. leprae* and *M. vaccae* in the same manner *in vitro* (table 2).

This was further confirmed in the *in vivo* experiments where the Swiss white mice infected subcutaneously with *M. vaccae* showed a dramatic reduction in F_c receptor expression by their peritoneal macrophages even after an extended period of 6 months (table 3).

Table 2. F_c receptor expression in macrophage cultures infected *in vitro* with *M. leprae* or *M. vaccae*

	EA rosetting (%)		
	1	2	3
Control	53	95	85
+ *M. leprae* (5 × 10⁶/culture)	38	56	60
+ *M. vaccae* (5 × 10⁶/culture)	39	60	36
+ *M. leprae* (3 × 10⁶/culture) ⎫ + *M. vaccae* (3 × 10⁶/culture) ⎭	23	25	56

Table 3. F_c receptor expression of mice sensitized with *M. vaccae in vitro.*

	EA rosetting (%)					
	1	2	3	4	5	6
Control	8	10	5	13	65	93
+ *M. leprae*	8	12	7	14	48	70

Mice 1–4 sacrificed after 6 months (mice 5 and 6 sacrificed after 1½ years).

Table 4. Macrophage–lymphocyte interaction of mice sensitized with *M. vaccae in vivo.*

		1	2	3	4	5
% Interaction	No. antigen	25	20	19	3	5
	M. leprae	38	54	31	18	15
	M. vaccae	30	33	27	19	12
% Macrophage with AFB	*M. leprae*	38	40	26	50	30
	M. vaccae	0	2	4	4	0

Mice 1–3 sacrificed after 6 months (mice 4–5 sacrificed after 1½ years).

Macrophage–lymphocyte interaction in Swiss white and C57Bl/6 mice

The interaction in response to *M. leprae* in spleen and lymph node was positive. However most striking was the clearance of *M. vaccae* in the interaction system. In contrast, the percentage of cells harbouring *M. leprae* was much higher (table 4).

Discussion

An analysis of the BCG field trials in Uganda and Burma by Stanford and his colleagues (Stanford *et al.*, 1981; Rook *et al.*, 1981) concluded that one of the factors influencing the protective efficacy of BCG was environmental mycobacteria. Sensitization to fast growing mycobacteria (*M. nonchromogenicum*, *M. vaccae*) was found to provide protection against leprosy. While *M. scrofulaceum* and possible other slow growers are antagonists of the protective effect of BCG.

On the basis of these observations they hypothesize that contact with environmental mycobacteria will induce the Listeria type or the Koch-type response and BCG vaccination will enhance it. Thus in those places where environmental species prime for the Listeria type of response subsequent BCG vaccination will afford good protection.

Our results of F_c receptor activity, macrophage–lymphocyte interaction and lymphocyte proliferation in the presence of L-lysate show that among the mycobacteria tested *in vitro*, *M. vaccae* appears to be the most successful as it consistently reverses all 3 parameters. These results support the observation of Rook *et al.* (1981) that exposure to *M. vaccae* or *M. nonchromogenicum* could lead to protection, while contact with *M. scrofulaceum* or *M. kansasii* did not result in protection.

An interesting outcome of our results is that BCG did not demonstrate any enhancement in EA rosetting or interaction values, however in the lymphocyte proliferation assays in addition to *M. vaccae*, BCG responses were also not inhibited by L-lysate and no suppressor factor(s) production was also noted.

It is important to note that in the interaction system 2 out of 5 of lepromatous patients could not be converted to positivity with ICRC and in the lymphocyte proliferative assay suppression in the presence of ICRC and L-lysate was evident also in 2 out of the 5 cases though in one case it was marginal. These figures are comparable to those obtained in clinical trials using ICRC (Deo *et al.*, 1981). Nevertheless F_c continued to remain negative. In contrast, *M. delhi* did not show any enhancement of reactivity both by the F_c and interaction systems though clinical trials appear promising (Chaudhury *et al.*, 1983). The existence of alternative mechanisms of immune activation cannot be overlooked.

Our results supply evidence that the mouse cannot be used as a model for vaccine studies though it may help in elucidating other aspects of the immune response to *M. leprae*. This is evident from the prolonged decrease of F_c receptor expression in *M. vaccae* infected mice and the inability of the splenic macrophages to clear *M. leprae*, while showing a concomitent clearance of *M. vaccae*. The observations put together highlight the potential use of *M. vaccae* as an immunomodulator and the report also describes 3 protocols that could be further developed as useful *in vitro* tests for screening of immunomodulating mycobacteria.

Acknowledgements

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Minimum inhibitory concentration of drugs against *Mycobacterium leprae* as determined by an *in vitro* assay

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Abstract. The observations that live *Mycobacterium leprae* after entry into cultured peritoneal macrophages from mice, reduced the EA rosetting macrophages, have been exploited to determine the minimum inhibitory concentration of diamino diphenyl sulphone and rifampicin. Diamino diphenyl sulphone showed a minimum inhibitory concentration of 0·028 μg/ml and rifampicin 0·11 μg/ml when given externally. However, there was accumulation of diamino diphenyl sulphone inside the macrophages. At an external concentration of 0·028 μg/ml the concentration inside the macrophage was 0·5 μg/ml. The minimum inhibitory concentration for diamino diphenyl sulphone in this assay system is higher by several folds and that for rifampicin is slightly lower, than what is reported earlier with mice foot pad experiments. The minimum inhibitory concentration reported in this assay system is quite close to what is observed for *in vitro* inhibition of *Mycobacterium lufu* with both the drugs.

Keywords. *Mycobacterium leprae*; minimum inhibition concentration; drugs; F_c receptor assay.

Introduction

Among the drugs that are in clinical use against leprosy the diamino diphenyl sulphone (DDS) and rifampicin are most widely used, either individually or as components of recently introduced multidrug therapy (WHO, 1982).

Due to nonavailability of an *in vitro* drug assay using *Mycobacterium leprae*, the minimum inhibitory concentration (MIC) has been reported using experimentally infected mice. The MIC for DDS has been determined as 0·003 μg/ml (Peters *et al.*, 1975) and for rifampicin as 0·3 μg/ml (Holmes and Hilson, 1972) based on the serum level. While this is an interesting and useful piece of information, less confidence is placed on these values in determining the dose of the drug given to the patients. The conventionally administered dose of 100 mg/day and 600 mg at a time, for DDS and rifampicin respectively, according to Ellard (1980), provides a peak serum level of 500 and 30 times the MIC. This is given perhaps, with the idea of avoiding development of drug resistant *M. leprae* in the patients. Thus the heavy dose of drug has no relationship to the MIC as determined in mice. It is thus useful to have a much more direct *in vitro* method of determining susceptibility of *M. leprae* to these drugs and the MIC.

Abbreviations used: DDS, Diamino diphenyl sulphone; MIC, minimum inhibitory concentration; SRBC, sheep red blood cells.

It has been demonstrated that the presence of live *M. leprae* inside the macrophages from human or mice, reduced the level of F_c receptor expressing cells. This was not obtained with killed *M. leprae* (Birdi *et al.*, 1983; Birdi and Antia, 1984; Mankar *et al.*, 1984). The basic conclusion derived from this study was that live *M. leprae* but not inactive *M. leprae* were able to alter the surface structure of macrophages. This surface structure alteration resulted in the reduction of F_c receptor expressing macrophages only in the presence of live *M. leprae*. Thus if drugs are active on *M. leprae*, then in the presence of the drug and *M. leprae*, F_c receptor expressing macrophages will not be reduced and the level will be as good as the control (Birdi and Antia, 1984; Mankar *et al.*, 1984).

In this report we present data indicating the MIC of DDS and rifampicin that would inactivate *M. leprae*, such an inactivation being determined using the above *in vitro* test system.

Materials and methods

DDS uptake by macrophages

Macrophages from the peritoneal cavity of Swiss white mice were obtained and cultured in Leighton tubes as described previously (Mankar *et al.*, 1984). Seventy two hours after distribution of macrophages, the drug DDS was added to the medium. After 3 days of exposure to the drug, the cells were harvested by scraping them with a rubber policeman. They were washed 3 times with saline (0·85 %) to remove extracellular DDS. After suspending the macrophages in 1 ml saline, the cell count was taken in a haemocytometer. The macrophages were then lysed by subjecting them to 8 cycles of freeze-thawing. Distilled ethyl acetate (AR grade) was added to extract DDS from the macrophages. The extraction was done thrice by adding 2 ml ethyl acetate each time. To keep the pH basic, 0·5 ml of 10 N NaOH was added during the first extraction. The mixture was vortexed each time to facilitate the extraction of DDS from the lysate to the ethyl acetate layer (Ellard and Gammon, 1969; Ozawa *et al.*, 1971; Peters *et al.*, 1981).

After separation of the solvent and aqueous phase in 20 min, the ethyl acetate phase was transferred to another tube with a pasteur pippette. To the ethyl acetate phase 0·5 gm of NaCl (AR grade) was added to remove water molecules carried during the extraction. The ethyl acetate phase was then allowed to separate from the NaCl which sedimented at the bottom. The ethyl acetate phase was removed and checked for fluorescence on a spectrofluorimeter (SFM23-Kontrcn). This assay method provided a sensitivity upto 2 ng/ml for DDS at wavelength settings of 285 nm for excitation and 350 nm for emission. A culture of macrophage, which had not been exposed to the drug otherwise treated exactly the same as the experimental served as control.

DDS (in ng)/10^6 macrophages is equal to a fluorescence level of 1×10^6 experimental macrophages, from which the fluorescence level of 1×10^6 control macrophages was subtracted. The quantitation was done using a standard curve (experimentally determined) of fluorescence units against increasing concentrations of DDS added to macrophage cultures.

Volume measurement

The macrophages exposed to DDS as described earlier were suspended in osmolar saline and a drop was placed on a wax coated slide. The macrophages which had settled, appeared rounded and uniformly circular under microscopic observation. The diameter of macrophages was measured using previously calibrated ocular micrometer. One hundred macrophages were scanned and the diameter was measured, to determine the average diameter. The volume of a macrophage was calculated using the formula. $V = 4/3\,\pi r^3$ where r is radius, which was determined as $10\,\mu m$ (table 1). Thus $V = 4/3 \times 3{\cdot}17 \times (10 \times 10^{-4})$. This worked out to be $4{\cdot}2 \times 10^{-9}$ cc for a single macrophage.

Determination of minimum inhibitory dose of DDS for M. leprae

The determination of minimum external dose necessary to inhibit the *M. leprae* inside the macrophage was done by the EA rosetting technique. Mouse peritoneal macrophage cultures were set and 72 h later the drug sulphone (Burroughs Wellcome India) was added in known concentrations. After exposure of the culture to the drug for 72 h, they were washed to remove the external DDS and were infected with *M. leprae* (armadillo derived). The extracellular drug was removed so as to avoid killing of *M. leprae* outside the cells. After the removal of extracellular DDS, there was very little diffusion of (5 % of internal level) cellular DDS to outside during the 72 h of incubation. *M. leprae* were phagocytosed by macrophages and were allowed to interact with the drug inside macrophages for 72 h (5×10^6 *M. leprae*/tube was the dose). On the day of termination all the cultures were washed to remove the excess bacilli and subjected to EA rosetting with antibody coated sheep red blood cells (SRBC) (Mankar *et al.*, 1984). *M. leprae* was prepared from infected armadillo tissue adopting the procedure of Ambrose *et al.* (1978). In all our experiments there was very little or no phagocytosis of SRBC by the macrophages.

Results

The radius of cultured macrophages was found to be $10\,\mu m$ as an average value determined from several experiments using different concentrations of DDS and 100 cells in each experiment (table 1). This radius was used to calculate the volume of the macrophages and the volume was determined to be $4{\cdot}2 \times 10^{-9}$ cc for a single macrophage. The macrophages mostly retained a uniform circular shape. To avoid experimental variation several experiments were done as stated above and the average value has been used.

The percentage of macrophages expressing F_c receptors was reduced in the presence of live *M. leprae*. In the presence of appropriate concentration of DDS in the incubation fluid, this F_c reduction was counteracted (table 2). At DDS concentrations of 14·2 ng/ml and 21·4 ng/ml, the level of F_c receptor expressing macrophages in the presence of live *M. leprae* was 24 ± 6 and 46 ± 7 % as compared to the control values of 49 ± 14 and 70 ± 1 % respectively. However concentrations of DDS at 28·5 ng/ml and above completely reversed the lowered level of F_c receptor containing macrophages

Table 1. Average radius of macrophage in culture while exposed to varying concentrations of DDS.

Conc. of DDS	No. of cells with radius of 9 μ	10 μ	11 μ	Average value (in μm)	
10 ng	21	69	9	9·78	
	16	55	29	10·13	
15 ng	17	61	22	10·05	
	18	62	20	10·02	
20 ng	19	65	16	9·97	Common
	16	65	19	10·03	Average
50 ng	12	52	36	10·20	value
	15	68	17	10·07	10 μ
100 ng	24	63	13	9·87	
	26	51	23	9·97	
1000 ng	22	35	43	10·21	
	19	40	41	10·22	

Total no. of cells counted was 100 in each of the experiment and two experiments were conducted at each concentration of DDS.

Table 2. Percentage of macrophage exhibiting EA rosetting in the presence of *M. leprae* and various concentrations of DDS.

DDS conc. (ng). Per tube	Per ml medium	% EA rosetting (mean + S.D.)* Control A	+ M.L. B	+ DDS C	+ DDS + M.L. D	+ M.L. (H.K.) E
10	14·2	49 ± 14†	24 ± 6	30 ± 8	31 ± 9	55 ± 13
15	21·4	70 ± 1	46 ± 7	55 ± 3	59 ± 3	70 ± 1
20	28·5	75 ± 5	48 ± 6	56 ± 5	76 ± 5	75 ± 5
50	71·4	72 ± 2	46 ± 8	60 ± 2	71 ± 2	72 ± 2
100	142·8	68 ± 3	42 ± 3	56 ± 9	70 ± 3	69 ± 5

* The value for each concentration of DDS is the mean of 4 experiments. P value is for data calculated for each concentration of the drug separately.
A–B, $P < 0.05$; A–C, $P < 0.05$; A–D, $P < 0.05$ upto 15 ng; A–E, $P > 0.05$; B–D, $P > 0.05$ upto 15 ng. Significance calculated by Student's t distribution test.
† A different sample of amboceptor (antibody) for SRBC was used in this set of experiment and so the values are lower for this concentration.

caused by live *M. leprae*. This indicated the drug induced inactivation of *M. leprae* at this concentration. The cultures using heat killed *M. leprae* showed the same rosetting ability as the control where no live *M. leprae* was added, the results being similar to those cultures treated with live *M. leprae* and concentration of DDS at 28·5 ng/ml or higher. The drug by itself, in the absence of *M. leprae* lowered the F_c receptor containing macrophages. This was noteworthy, but as we were looking for a reversal of the lowered F_c receptor level to higher level in the presence of the drug and *M. leprae* the effect of drug by itself is of little significance in this connection.

Table 3 presents data that were calculated to determine the DDS concentration of macrophages ($\mu g/ml$ of the cells) using various concentrations of the drug in the culture. It is clearly seen that the concentration of the drug inside the macrophages increases with increasing concentration of the drug that was provided outside the cells.

From table 2 it is clear that at 28·5 ng/ml (external concentration) DDS inactivates *M. leprae*, so as to block its effect on the membrane of the macrophages and thereby prevent the reduction of F_c receptor bearing macrophages. At this concentration of 28·5 ng/ml, the level of DDS inside the macrophages is 0·50 $\mu g/ml$, the range varying from 0·3 μg–0·6 $\mu g/ml$.

Data presented in table 4 show that a minimum concentration of 114 ng/ml of rifampicin (Sigma, USA) is needed to inactivate the *M. leprae* inside the macrophages. This was shown by the restoration of the normal level of F_c receptor macrophages when this concentration of the drug was used in the presence of live *M. leprae*.

Table 3. Level of DDS inside macrophages (as $\mu g/ml$) on exposure to various amounts of the drug.

External concentration ng/tube	ng/ml	DDS/10^6 macrophage* (ng)	DDS/one* macrophage (fg)	DDS in macrophage* (vol.) ($\mu g/ml$)
10	14·2	1·5	1·5	0·37±0.02
20	28·5	2·15	2·15	0·50±0·09
50	71·4	6·1	6·1	1·44±0·24
100	142·8	8·0	8·0	1·89±0·21
200	285·6	25·3	25·3	6·02±1·09
500	714·0	35	35	8·36±1·6

* Value determined as mean ± S.D. from 4 experiments at each concentration of DDS. The volume of macrophage used for calculation was $4·2 \times 10^{-3}$ cc/10^6 macrophage.

Table 4. Percentage of macrophages exhibiting EA rosetting in the presence of *M. leprae* and at various concentration of rifampicin.

RFP conc. (ng) Per tube	(per ml) (ng)	Control A	%EA rosetting (Mean ± S.D.)* + M. leprae B	+ RFP C	+ RFP + M. leprae D	M. leprae (H.K) E
20	28·5	59±3	31±3	43±7	49±7	59±3
50	71·4	65±3	38±4	51±6	52±5	66±4
80	114·2	65±6	34±3	51±6	63±4	64±5
100	142·8	62±4	36±5	53±4	62±5	62±1

* 3 experiments were carried out for each concentration and the mean ± S.D. is presented. 5×10^6/Leighton tube of *M. leprae* was used.
Significance of difference (Student's *t* distribution test). *P* value is for data under each concentration of rifampicin used.
A–B, *P* < 0·05; A–C, *P* < 0·05; A–D, *P* < 0·05 upto 50 ng only; A–E, *P* > 0·05; B–D, *P* > 0·05 upto 50 ng only.

Discussion

The MIC for DDS using the F_c receptor assay system appears to be 28 ng/ml of the medium. Satish and Nath (1981) and Nath *et al.* (1982) in their experiments had shown that at 10 ng/ml level of DDS, [^{3}H]-thymidine incorporation by *M. leprae* could be considerably reduced and even 3 ng/ml was also shown to be active to some extent. This was explained as being in agreement with the MIC for DDS reported from mice experiments. However the basic fact that the drug accumulated inside the macrophages was not taken into consideration, which is an important feature in any assay using host cells. At an outside concentration of 10 ng/ml the expected concentration inside the macrophages is 0·30–0·38 μg/ml as seen in our experiments.

When the concentration of DDS in the culture was varied from 10–500 ng there was an increasing level of DDS inside the macrophages, although a linear relationship was seen only in the lower concentration range of 10–100 ng levels (table 3). This lower range is quite satisfactory for our experimental purpose; since at these levels DDS is active.

Seydel and Wempe (1982) had shown that *M. lufu* which appears as the best model organism to study the action of DDS, the inactivation and growth inhibition occurred at an MIC of 0·03–0·05 μg/ml and this is quite close to the external DDS concentration observed in our assay system (0·028 μg/ml).

DDS has the ability to bind to proteins and some of the internal DDS could be protein bound. Since we extract DDS with ethyl acetate it is possible that the protein-bound fraction is also included in the estimation and therefore the active free DDS concentration inside *M. leprae* may be even lower.

In mice foot pad experiments the MIC determined on the basis of the serum level of DDS can give only part of the information, since the bacteria that are to be killed are in the foot pad and therefore the accumulated level of DDS in the local tissue is the most relevant parameter. The accumulated level will depend on storage and degradation of the drug in the tissues. It has been reported that the tissue level is likely to be 2-fold that of the serum level. Gordon *et al.* (1974) have reported that the serum level of DDS, when mice were given 0·002 % DDS in the diet, was 180 ng/ml and in the tissue it was 1·6 times higher. Shepard (1967) calculated an MIC of 0·01–0·03 μg/ml and Ozawa *et al.* (1971) reported it to be 2·5–10 ng/ml. Ellard *et al.* (1971) concluded that the MIC of DDS to *M. leprae* is less than 10 ng/ml, based on the serum level. Holmes and Hilson (1972) have shown that the serum value at 0·0001 % of DDS in diet was between 0·01–0·03 μg/ml. This is similar to the value recorded by us. The often quoted report of Peters *et al.* (1975) indicating an MIC value of 0·003 μg/ml in rats and by Levy and Peters (1976) in mice, is in close agreement with the value obtained in the present study.

The level of an MIC of 28 ng/ml needed in our test system may probably be due to the fact that our experiments are limited to only 72 h exposure of the bacteria to the drug compared to the exposure of *M. leprae* in mouse foot pad for several weeks to the lowest reported level of 6 ng/ml (or perhaps higher) of the drug in the tissue before any viability is checked. Seydel and Wempe (1982) using the cell free enzyme system showed that the concentration required to lower the folate synthesizing enzyme activity from *M. lufu* by 50 % with DDS was 0·084 μg/ml (0·42 μM). They also pointed out that the DDS concentration needed for *in vitro* inactivation of the enzyme is definitely much

higher than 0·003 μg/ml reported as the MIC. Thus it is quite probable that the accumulated tissue level of DDS acts on *M. leprae* in mice foot pad experiments.

In patients receiving 100 mg/day, DDS reached peak serum levels of 2·5–6 μg/ml (Modderman *et al.*, 1983). Thus the serum level is atleast 5–12 times more than the MIC determined by the present method. So it can be argued that a level even lower than 100 mg/day should be quite sufficient as a therapeutic dose. It could also be mentioned that sulpha drugs are always given at a dose level capable of completely inactivating the folate synthesizing enzyme.

In comparison to DDS, rifampicin directly blocks the multiplication of bacteria and it is bactericidal. The MIC determined in the mice was 0·3 μg/ml (Holmes and Hilson, 1972). In our system we needed a minimum external dose of 0·114 μg/ml (80 ng/Leighton tube) to inactivate *M. leprae* inside the macrophages.

It has not been possible to estimate the concentration of rifampicin inside the macrophages when a concentration of 0·114 μg/ml (80 ng/Leighton tube) is given. The sensitivity of the bioassay is only up to a level above 0·1 μg/ml. Thus the level of accumulation cannot be more than 0·1 μg/ml and this needs further investigation. It is possible that there is accumulation of rifampicin inside the macrophages and the actual concentration of the drug to which *M. leprae* inside the cells is exposed could be higher than the external concentration. Using *M. lufu* as test organism, the MIC of rifampicin is reported to be 0·25 μg/ml by Seydel *et al.* (1982).

The potentiality of determining the MIC of a drug using the F_c receptor assay, can also help us to determine the synergistic effect of one drug in the presence of another. Such studies have been completed using this assay system with two drugs DDS and Brodimoprim (Seydel *et al.*, 1983; Jagannathan and Mahadevan, 1985).

Acknowledgements

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Mechanism of action of aflatoxin B₁

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Abstract. The inhibitory effects of aflatoxin B₁ were found to be related to the gram character in procaryotes, used in this study. Ethylene diamine tetra chloroacetic acid (0·05 % w/v) or Tween-80 (0·05 % v/v) addition accentuated the aflatoxin B₁ growth inhibition in *Salmonella typhi* and *Escherichia coli* at different pH values. The inhibition of lipase production was only 5–20 % in *Pseudomonas fluorescence* ca. 25–48·% in *Staphylococcus aureus* and *Bacillus cereus* at different aflatoxin B₁ concentrations (4–16 μg/ml). However, inhibition of α-amylase induction was complete in *Bacillus megaterium* whereas the inhibition was partial in *Pseudomonas fluorescence* (27–40%) at 32 μg aflatoxin B₁ concentration. An increase in leakage of cell contents and decreased inulin uptake were observed in toxin incubated sheep red blood cell suspension (1 %) with increased aflatoxin B₁ concentration.

Keywords. Aflatoxin B₁; α-amylase; cell content leakage; enzyme induction/inhibition; lipase procaryotes.

Introduction

Since 1961, when aflatoxicosis claimed many deaths in turkey poults, pheasants, ducklings and chicks (Asplin and Carnaghan, 1961; Blount, 1961; Carnaghan and Allcroft, 1962; Sargeant *et al.*, 1961; Spensley, 1963), both procaryotes and eucaryotes have been extensively used for detection and quantification of aflatoxins (Arai *et al.*, 1967; Butler and Barnes, 1963; Childs and Legator, 1966; Gablike *et al.*, 1965; Lillehoj and Ciegler, 1968; Vosdingh and Nefe, 1974). Various aflatoxin B₁ effects included aberrant cells in *Bacillus megaterium* (Beuchat and Leochowich, 1971), decrease in cell number, protein concentration, RNA and DNA contents (Gabliks *et al.*, 1965), binding to cells with and without nucleic acids (Lillehoj and Ciegler, 1968), lysogenic and mutagenic properties (Legator, 1966), inhibition of respiration (Nezval and Bosenberg, 1970), inhibition of enzyme biosynthesis (Anand, 1971; Lillehoj and Ciegler, 1970; Black and Altschul, 1965) and alteration of enzyme levels and activity in tissues (Brown and Abrams, 1965). The exact mode of aflatoxin B₁ action still remains unknown. In the present study the action of aflatoxin on gram positive and gram negative bacteria has been delineated.

Materials and methods

Aspergillus flavus NRRL 2999, a potent producer of aflatoxin (Hesseltine *et al.*, 1966) procured from Peoria, USA, was used for aflatoxin production using rice as substrate (Shotwell *et al.*, 1966). Aflatoxin B_1 was purified and quantitated following the method of Nabney and Nesbitt (1965).

Effect on procaryotic cells

The test organisms included *Salmonella typhi* (5 strains), *S. paratyphi* A and B (5 strains each), *S. typhimurium*, *S. enteritidis*, *S. anatum*, *S. gallinarum* (2 strains), *S. poona*, *S. choleraesuis*, *Bacillus megaterium* (5 strains), *B. subtilis* (5 strains), *S. aureus* (10 strains), *Escherichia coli* (8 strains). These were obtained from Departmental Culture Collection and maintained on nutrient agar slants at 4°C, subcultured every month.

Effect on growth

An appropriate amount of aflatoxin B_1 (20 µg/ml) was added in presterilized trypticase soy broth (5 ml/tube). The tubes were incubated with 0·1 ml of diluted (1:100) 24 h culture of test organism and incubated at 37°C for 24 h. Scoring from $-$ to $+++++$ indicates the range, from no growth to maximum growth. In another experiment, growth was monitored by measuring absorbance increase at 540 nm at 4 h intervals during incubation. The aflatoxin B_1 (20 µg/ml) growth inhibition was also studied in the presence of Tween-80 (0·05 % v/v) or ethylene diamine tetra chloroacetic acid (EDTA) (0·05 % w/v) at different pH values (pH 6·0–8·0) in *E. coli* and *S. typhi*. The per cent inhibition was calculated as given below:

$$PI = \frac{A_{540} \text{ control} - A_{540} \text{ test}}{A_{540} \text{ control}} \times 100$$

Effect on enzyme biosynthesis

Strains of *P. fluorescence* and *B. megaterium* having inducible α-amylase were induced following the method of Averner and Klein (1963). Samples were removed at 1 h intervals for 4 h, centrifuged (10,000 *g* for 15 min) and the supernatant assayed for amylase activity (Bernfeld, 1955).

   S. aureus, *B. cereus* and *P. fluorescence* were used to study the effect of aflatoxin B_1 (4–16 µg/ml) on lipase production. A heavy suspension of these organisms (1·0 absorbance at 540 nm) was made in nutrient broth and incubated for 24 h at 37°C. The cells were centrifuged and supernatant assayed for lipase activity (Vadehra and Harmon, 1969).

Effect on permeability

Inulin uptake and leakage of cellular contents were determined in 1 % sheep red blood cell (SRBC) suspension in normal saline (0·85 % NaCl). The cells were incubated with aflatoxin B_1 (20–80 µg/ml) and inulin (8 µg/ml) at 37°C for 2 h. The cells were centrifuged at the end of incubation and supernatant assayed for residual inulin

(Corcoran and Page, 1939) and haemoglobin leakage at 555 nm. The absorbance of supernatant was also measured at 430 nm (for components other than haemoglobin).

Results

Aflatoxin B_1 (20 $\mu g/ml$) inhibited growth in 25 strains out of 48 strains (gram positive and gram negative) used in this study. The inhibited strains included *S. aureus* (10 strains) and 5 strains each of *B. megaterium, B. cereus* and *B. subtilis* (table 1). However, a slight decrease in growth rate in the presence of aflatoxin B_1 (20 $\mu g/ml$) was observed in trypticase soy broth (pH 7·3) when the test organisms were *E. coli* and *S. typhi* (figure 1). The growth decreased further on addition of Tween-80 or EDTA (0·05 %) at various pH values. An overall decrease in growth rate was observed at all pH values (pH 6·0–8·0) in the presence of aflatoxin B_1 and EDTA or Tween-80 indicating synergistic inhibitory effects of surfactants with aflatoxin B_1 on *S. typhi* and *E. coli*.

Table 1. Effect of aflatoxin B_1 (20 $\mu g/ml$) on growth.

Organism			Visual comparison of growth
Salmonella typhi	TN	6416	+ + + +
S. typhi	WHO	68.7	+ + + +
S. typhi	AP	7039	+ + + +
S. typhi	AP	7023	+ + + +
S. typhi			+ + + +
S. paratyphi A	TN	6420	+ + + +
S. paratyphi B	WHO	6393	+ + + +
S. typhimurium	TN	6417	+ + + +
S. enteridis	WHO	9398	+ + + +
S. anatum	TN	6437	+ + +
S. gallinarum	MY	6905	+ + + +
S. gallinarum	MY	6910	+ + + +
S. poona	MP	6788	+ + + +
S. welterberg	MP	6829	+ + + +
S. choleraeruis	Cal	7064	+ + + +
E. coli		1040	+ + + +
E. coli		4083	+ + + +
E. coli	H	30	+ + + +
E. coli	W		+ + +
E. coli	K	10	+ + +
E. coli	K	12	+ + + +
E. coli	J	32	+ + + +
E. coli		410G	+ + +
B. megaterium		(5 strains)	−
B. subtilis		(5 strains)	−
B. cereus		(5 strains)	−
S. aureus		(2 strains)	+
S. aureus		(8 strains)	−

− = No growth   + = Extent of growth
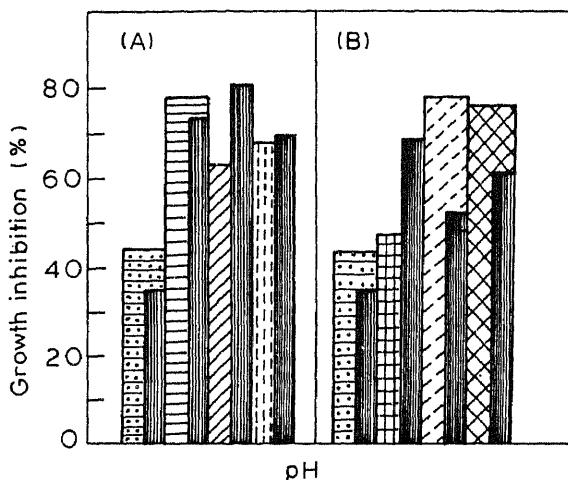
Figure 1. Aflatoxin B_1 effect on growth of *E. coli* in the presence of (A) EDTA (0·05 %) at pH 6·0 (⊟), pH 7·0 (▨) and pH 8·0 (▥) or (B) Tween-80 (0·05 %) at pH 6·0 (▦), pH 7·0 (▨) and pH 8·0 (▨) and in the absence at pH 7·3 (▨) in trypticase soy broth at 37°C. The values for *S. typhi* growth inhibition have been represented by solid bars (▥).

The aflatoxin B_1 effects were also studied on enzyme activity/biosynthesis in both kinds of organisms. The extent of enzyme inhibition (production/induction/activity) was much higher in gram positive organisms than in gram negative organisms (figure 2). The lipase production was approximately 50 % as compared to control in *S. aureus* and *B. cereus* at 16 μg/ml aflatoxin B_1 concentration whereas, *P. fluorescence* could show 80 % activity at this concentration. The extent of amylase inhibition varied with toxin concentration (8–32 μg/ml) and the time of incubation. The inhibition of enzyme induction ranged from 45·5–100 % in *B. megaterium* and 29·2–72·7 % in *P. fluorescence* at different aflatoxin B_1 concentrations (8–32 μg/ml). At all concentrations of toxin, the inhibition was always higher in *B. megaterium* than in *P. fluorescence* (figure 3). A continual decrease in inulin uptake (33–100%) and an increase in leakage of cell constituents were observed with increased amount of toxin (20–80 μg/ml) in suspending medium (table 2). The centrifuged supernatant of toxin incubated cell suspension showed increase in absorbance at 555 nm (for haemoglobin) and 430 nm (other than haemoglobin).

Discussion

DNA binding and inhibition of nucleic acid synthesis is the most common mechanism suggested for aflatoxin B_1 action (Clifford and Rees, 1967; Stark, 1980; Wragg *et al.*, 1967). In the present study gram positive organisms were comparatively more susceptible to aflatoxin B_1-mediated growth inhibition than gram negative organisms (table 1). The above observation is in confirmation with the survey of Burmeister and Hesseltine (1966). This indicates the involvement of cell wall and or cell membrane in the action of aflatoxin B_1. The observation leading to formation of aberrant cells in *B. megaterium* (Beuchat and Lechowich, 1971) is also in agreement with the above
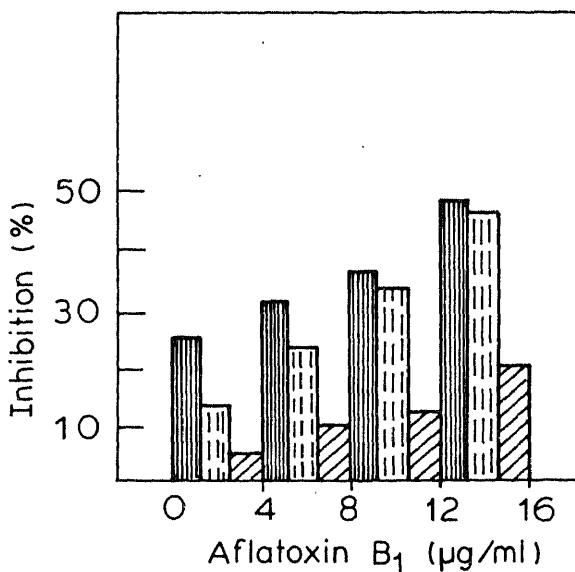
Figure 2. Effect of aflatoxin B₁ (4–16 μg/ml) on lipase production in *S. aureus* (▥), *B. cereus* (▥) and *P. fluorescence* (▨) at 37°C.
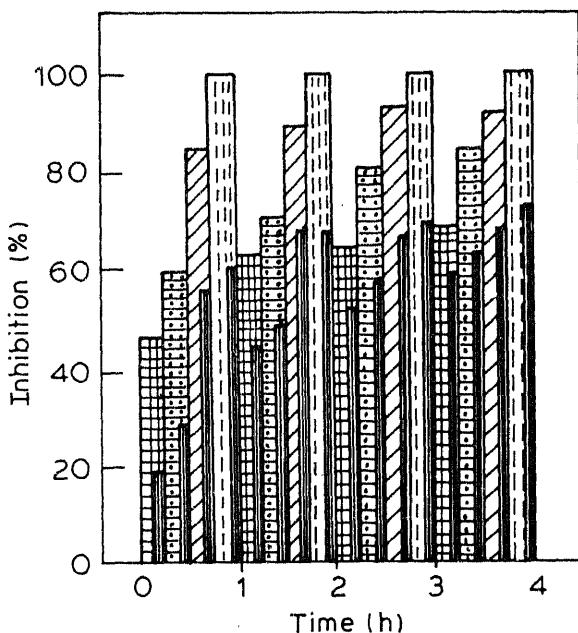
Figure 3. Effect of aflatoxin B₁ (8–32 μg/ml) on α-amylase induction. (▦), (▤), (▨), and (▦) represent α-amylase activity at 8, 16, 24 and 32 μg/ml aflatoxin B₁ in *B. megaterium* at 1–4 h of incubation. Values for *P. fluorescence* have been shown in solid bars (■).

Table 2. Effect of aflatoxin B_1 (20–80 μg/ml) on intracellular contents' leakage and inulin uptake in SRBC (1 %).

Aflatoxin conc.	Decrease in inulin uptake (%)	Absorbance of intracellular contents	
		555 nm	430 nm
20	32·7	0·110	0·340
40	38·5	0·150	0·470
60	51·9	2·000	2·000
80	100·00	3·000	3·000

hypothesis. This would indicate either differences in binding or the accessibility of aflatoxin B_1 to the effector site. Earlier work done in this laboratory found protoplasts more susceptible to aflatoxin B_1 than the parent organism, despite the fact, that the former bound less aflatoxin B_1 (Tiwari *et al.*, 1984). Hence, the cell wall appears to be a barrier to the action of aflatoxin B_1 which inference is also supported by accentuated inhibitory effects of aflatoxin B_1 in the presence of Tween-80 or EDTA in this study. EDTA, a chelator is known to affect permeability resulting in the release of cell wall polysaccharides with little or no loss of other cell components (Leive, 1968). The inhibition of enzyme bio-synthesis/activity by aflatoxin B_1 is also in agreement with earlier studies (Anand, 1971; Lillehoj and Ciegler, 1970; Black and Altschul, 1965). However, the extent of inhibition was found to be related to gram character in this study. The inhibition was more in gram positive organisms. This information along with the conclusions of low oxygen uptake (Nezval and Bosenberg, 1970) and inhibition of electron transport flow by aflatoxin B_1 (Doherty and Campbell, 1972) indicate the cell membrane to be the target for aflatoxin B_1 action. The decreased inulin uptake and an increased leakage of cell constituents observed in SRBC with increase in aflatoxin B_1 concentration further confirm the cell membrane as a target site. This finding is in agreement with an earlier report in procaryotes (Tiwari *et al.*, 1985). The decreased amino acid uptake (Clifford and Rees, 1967) and the changes in nucleous and disappearance of nucleoli (Gabliks *et al.*, 1965) reported earlier, can now be explained considering cell membrane as the primary target for aflatoxin action (Lillehoj and Ciegler, 1968). Therefore, the loss of cell membrane functions would be reflected in terms of the parameter used in the study *viz.* decrease in protein, nucleic acid content, decrease in the cell number (Gabliks *et al.*, 1965; Wragg *et al.*, 1967) and decrease in enzymes biosynthesis (Anand, 1971; Lillehoj and Ciegler, 1970; Black and Altschul, 1965). Hence, it is concluded that aflatoxin B_1 inhibitory effects in procaryotes are related to their gram character and the differences in susceptibility are because of an easy access of aflatoxin B_1 to the target site. The cell membrane appears to be the primary target for aflatoxin B_1 action.

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Transmission of male recombination, segregation distortion and sex-ratio imbalance in *Drosophila melanogaster*

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Abstract. From the first test cross progenies of control (no larval transfers; no ethyl methanesulphonate), physical stress (two larval transfers; no ethyl methanesulphonate) and 0·75% ethyl methanesulphonate (two larval transfers; 0·75% ethyl methanesulphonate)-treated F_1 (Oregon K + /*dumpy black cinnabar, dp b cn*) males of *Drosophila melanogaster*, respectively, 6, 10 and 52 wild-looking first test cross males were again test crossed to obtain second generation. The overall percentages of male recombination detected in the second test cross progenies, in the three sets of experiments, were statistically the same as those in the first test cross progenies. Thus the enhanced male recombination caused by physical stress (with or without ethyl methanesulphonate) was transmitted to next generation. Non-reciprocal male recombination was observed in *dp b* but not in *b cn* region in both first and second test cross progenies. Three abnormalities, (i) production of wild-type flies in majority over *dp b cn* type, (ii) Non-Mendelian segregation at *dp b* and *cn* loci and (iii) sex-ratio differences for *dp b cn* and + *b cn* types observed in test cross progenies of F_1 males of *Drosophila melanogaster* were transmitted to next generation when induced with 0·75% ethyl methanesulphonate but not when these abnormalities were induced with physical stress. The data suggest possible association of non-reciprocal male recombination, segregation distortion and sex-ratio imbalance in *Drosophila melanogaster*. In fact these may be representing different aspects of the same phenomenon.

Keywords. *Drosophila melanogaster*; non-reciprocal recombination; male recombination; segregation distortion; sex-ratio.

Introduction

Recombination, normally considered to be absent in males of *Drosophila melanogaster* (Morgan and Bridges, 1919), can nevertheless be induced with a wide variety of physical and chemical agents (Thapar, 1982; Mohindra, 1984). With ethyl methanesulphonate (EMS) and chloroquine phosphate, using 3 genetic markers – *dumpy(dp)*, *black(b)* and *cinnabar(cn)* in *D. melanogaster*, Miglani and Thapar (1983a) reported induction of non-reciprocal male recombination in *dp b* but not in *b cn* region. In addition, negative chromosomal interference and segregation distortions were also noted. In the present experiments, the nature and distribution of spontaneous, physical stress and EMS-induced male recombination events, segregation patterns and sex-ratio have been studied for two consecutive generations to provide information on the transmission of these phenomenon.

Abbreviations used: EMS, Ethyl methanesulphonate; *dp, dumpy*; *b, black*; *cn, cinnabar*; TC_1, first test cross; TC_2, second test cross; CD, coefficient of dispersion; SD, segregation distortion.

Materials and methods

Two stocks of *D. melanogaster*, a standard wild-type laboratory strain (Oregon-K) and a 'mutant' stock homozygous for 3 second chromosome recessive markers, *dumpy (dp*: 2–13·0) *black (b*: 2–48·5) and *cinnabar(cn*: 2–57·5) were used. EMS (Sigma Chemical Co., Batch No. 89c-0439) was used as a probe. Miglani and Thapar (1983b) determined 0·75 % EMS to be LD_{50} in second one-third part of larval life of *D. melanogaster* at 25°C. This dose of EMS was found to be most efficient in inducing recombination in males of *D. melanogaster* (Miglani and Thapar, 1983c). It was, therefore, decided to treat the developing F_1 (Oregon-K + /*dp b cn*) individuals with 0·75 % EMS, mixed with food in ratio 1 : 9 in the second one-third part of larval life following the method of Miglani and Thapar (1983b). Accordingly, after 54 h of egg deposition at 25°C, the developing F_1 larvae were flushed out with distilled water and then physically transferred with a camel hair brush on to the medium with or without probe solution. After allowing the larvae to feed for 32 h, they were again flushed out and transferred on to the standard food medium. Each untreated or EMS-treated larva, thus, underwent two transfers referred to as 'physical stress' in this report. A control experiment was run simultaneously with the above experiments where neither EMS was added to the food nor usual two transfers of larvae were done. In the present investigation, the following 3 sets of experiments were performed:

Set 1: No transfers, no EMS (control).
Set 2: Two transfers; no EMS (physical stress).
Set 3: Two transfers; 0·75 % EMS (0·75 % EMS).

A two-day old wild-looking F_1 (having probable genotype (Oregon-K + /*dp b cn*)) male was crossed with 3 *dp b cn* females to get the first test cross progeny (TC_1). Every TC_1 adult was screened for sex and phenotype. A few of the wild-looking (probably + /*dp b cn*) TC_1 males were again test crossed to obtain the second test cross progenies (TC_2) on standard food medium. All the TC_2 adults were studied for sex and phenotype.

In order to test the difference in frequencies of recombination recorded in the progenies of F_1' and TC_1 males from control, physical stress and 0·75 % EMS experiments, z-test was used (Gupta, 1980). Chi-square test was used to determine whether or not any two complementary products or males and females in a particular phenotype fitted into the desired ratio (Gupta, 1980). The χ-square values based on poisson distribution of the number of recombinants observed, were calculated following the Snedecor–Irwin method (Snedecor, 1956). The coefficient of dispersion values were calculated using the method of Sokal and Rohlf (1969).

Results

The TC_1 progenies of 14, 15 and 39 randomly selected *D. melanogaster* F_1 (Oregon-K + /*dp b cn*) males from control, physical stress and 0·75 % EMS experiments respectively, comprised of 3375, 5952 and 13887 individuals. Number of males and females recovered for each phenotype in TC_1 progenies is given in table 1. From these TC_1

Table 1. Pooled test cross progenies of F_1 and TC_1 wild looking males of *D. melanogaster.*

Phenotype	Control		Physical stress		0·75% EMS	
	M	F	M	F	M	F
Pooled TC_1 progeny from F_1 males						
dp b cn	749	803	1141	1404	3140	3347
+ + +	942	872	1643	1657	3398	3766
+ *b cn*	5	1	40	10	146	16
dp + +	3	0	2	6	20	26
+ + *cn*	0	0	0	0	4	6
dp b +	0	0	1	2	12	8
+ *b* +	0	0	0	0	1	0
dp + *cn*	0	0	0	0	0	0
Total:	1699	1676	2873	3079	6718	7169
M + F	3375		5952		13887	
Pooled TC_2 progeny from TC_1 males						
dp b cn	202	208	349	362	1944	2154
+ + +	248	210	341	377	2377	2427
+ *b cn*	0	0	7	4	77	27
dp + +	0	0	2	1	13	6
+ + *cn*	0	0	0	0	2	0
dp + +	0	0	0	0	1	0
+ *b* +	0	0	0	0	0	0
dp + *cn*	0	0	0	0	0	0
Total:	450	418	699	744	4414	4614
M + F	868		1443		9028	

M, Males; F, Females.

progenies, 6, 10 and 52 wild-looking males from control, physical stress and 0·75% EMS experiments, respectively, yielded 868, 1443 and 9028 flies in the TC_2 progenies on the standard food medium (table 1). The TC_1 and TC_2 progenies in the 3 experiments were compared for frequency of recombination, ratio of complementary products, segregation patterns and sex-ratio.

The F_1 males of *D. melanogaster* in control experiment, yielded 0·267% recombination (table 2) which was non-significant statistically. The overall frequency of recombination in the F_1 males of physical stress experiment (1·125%) was higher ($P < 0·001$) than that in the F_1 males of control experiment. A higher ($P < 0·01$) frequency of recombination was observed in F_1 males from 0·75% EMS experiment (1·706%) than that in F_1 males from physical stress experiment. No recombinant appeared in the progenies of control TC_1 males. The frequency of recombination in pooled TC_2 progeny of TC_1 males from physical stress experiment (0·97%) was significant ($P < 0·01$) as compared to the control. The TC_1 males from 0·75% EMS experiment yielded 1·395% recombination in TC_2, which was not significantly different

Table 2. Certain general comparisons in test cross progenies of F₁ and TC₁ (wild looking) males of *D. melanogaster*.

Comparison of	F₁ males			TC₁ males		
	Control	Physical stress	0·75% EMS	Control	Physical stress	0·75% EMS
No. males tested	14	15	39	6	10	52
Population size	3375	5952	13887	868	1443	9028
No. males producing recombinants	5	12	32	0	3	29
No. recombinants recovered	9	67	236	0	14	126
Per cent recombination in region:						
dp b	0·267	1·075	1·483	0	0·97	1·362
b cn	0	0·05	0·223	0	0	0·033
No. (per cent) males producing phenotype:						
dp b cn	14(100)	15(100)	39(100)	6(100)	10(100)	52(100)
+ + +	14(100)	15(100)	39(100)	6(100)	10(100)	52(100)
+ b cn	4(28·6)	12(80·0)	31(79·5)	0	3(30)	29(55·8)
dp + +	1(7·1)	2(13·3)	12(13·8)	0	1(10)	5(9·6)
+ + cn	0	0	6(15·4)	0	0	2(3·8)
dp b +	0	1(6·7)	7(18·0)	0	0	1(1·9)
+ b +	0	0	1(2·6)	0	0	0
dp + cn	0	0	0	0	0	0
No. of males producing various no. of recombinant classes:						
0	9(64·3)	3(20·0)	7(17·9)	6(1·0)	7(70·0)	23(44·2)
1	5(35·7)	10(66·6)	19(48·7)	0	2(20·0)	24(46·2)
2	0	1(6·7)	6(15·4)	0	1(10.0)	2(3·8)
3	0	1(6·7)	3(7·7)	0	0	3(5·8)
4	0	0	3(7·7)	0	0	0
5	0	0	1(2·6)	0	0	0
Maximum no. recombinants recovered in male	2	20	38	0	8	15
χ-Square values	3·489	5.89×10^{5a}	1.45×10^{16a}	0	1.1×10^{3a}	2.5×10^{6a}
Coefficient of dispersion values	1·468	7·29	7·04	0	4·46	1·39

Figures within parentheses indicate percentages. [a]$P < 0.001$ (see text for basis of calculation).

from that in TC_1 males from physical stress experiment. The overall frequencies of recombination revealed in control experiments by F_1 males (in TC_1 progeny) and TC_1 males (in TC_2 progeny) were not significantly different. Similar was the situation in physical stress and 0·75 % EMS experiments.

On the basis of number of F_1, or TC_1 males of *D. melanogaster* producing a particular phenotype (table 2), it was observed that in all the experiments all F_1 and TC_1 males produced the two parental phenotypes (+ + + and *dp b cn*) in their test cross progenies. The percentages of F_1 males producing a particular type of recombinant were higher than those of the TC_1 males producing the same recombinant phenotype, in all the experiments conducted.

The percentages of flies producing no recombinants among F_1 males were lower than those among TC_1 males (table 2). On the contrary, the percentages of individuals producing one or more recombinant classes among F_1 males were higher than those among TC_1 males.

The F_1 and TC_1 males of *D. melanogaster* were separately distributed in various classes on the basis of total number of recombinants recovered in the progeny of a particular male. The frequency distributions of F_1 and TC_1 males studied in control, physical stress and 0·75 % EMS experiments are given below. The figure preceding a hyphen represents the total number of recombinants recovered in test cross progeny of a particular male and the one followed by hyphen reveals the number of males in that class.

F_1 males

         Control (14 males): 0–9; 1–1; 2–4.
         Physical stress (15 males): 0–3; 1–3; 3–4; 4–2; 10–1; 14–1; 20–1
         0·75 % EMS (39 males) 0–7; 1–5; 2–6; 3–1; 4–2; 5–7; 6–1; 8–1; 10–1; 11–1;
         14–1; 15–2; 16–1; 17–1; 20–1; 38–1.

TC_1 males

         Control (6 males): 0–6
         Physical stress (10 males): 0–7; 3–2; 8–1.
         0·75 % EMS (52 males): 0–23; 1–7; 2–8; 3–2; 4–3; 5–2; 9–1; 10–2; 11–2; 12–1;
         15–1.

Expected poisson frequencies were calculated from the above frequency distribution, including even the unrepresented classes. The χ-square values, calculated on the basis of differences between the observed and expected frequencies were significant ($P < 0.001$) from F_1 and TC_1 males from physical stress and 0·75 % EMS experiments (table 2).

The coefficient of dispersion (CD) values, calculated from variance and mean of observed frequencies, for F_1 and TC_1 males of control, physical stress and 0·75 % EMS experiments, were greater than one, except for TC_1 males of control experiment where CD was zero (table 2).

The number of *D. melanogaster* flies appearing in parental (+ + + *versus dp b cn*) and recombinant (+ *b cn versus dp* + + ; + + *cn versus dp b* + ; + *b* + *versus dp* + *cn*) pairs of complementary classes in F_1 and TC_1 males of control, physical stress

and 0·75% EMS experiments were tested for 1:1 ratio (table 3). Only those complementary products that deviated from 1:1 ratio in a particular experiment are mentioned. The pooled TC_1 progenies from control, physical stress and 0·75% EMS experiments produced $+ + +$ flies in overwhelming majority ($P < 0.001$) over *dp b cn* type. The pooled TC_2 progeny from 0·75% EMS experiment revealed the same trend. The TC_1 and TC_2 progenies from physical stress and 0·75% EMS experiments produced $+ b cn$ flies in convincing majority ($P < 0.001$) over its complementary type *dp + +*.

Table 3. Comparison of complementary products, segregation pattern and sex-ratio in test cross progenies of F_1 and TC_1 (wild-looking) males of *D. melanogaster*.

	F_1 males			TC_1 males		
Comparison of	Control	Physical stress	0·75% EMS	Control	Physical stress	0·75% EMS
Complementary products (1:1 ratio)						
dp b cn versus $+ + +$	$<^c$	$<^c$	$<^c$	n	n	$<^c$
$+ b cn$ versus $dp + +$	n	$>^c$	$>^c$	NR	$>^b$	$>^c$
$+ + cn$ versus $dp b +$	NR	n	n	NR	NR	n
$+ b +$ versus $dp + cn$	NR	NR	n	NR	NR	NR
Segregation pattern (wild type vs. mutant allele) 1:1						
dumpy	d^c	d^c	d^c	m	m	d^c
black	d^c	d^c	d^c	m	m	d^c
cinnabar	d^c	d^c	d^c	m	m	d^c
Sex-ratio (1:1) for phenotype						
dp b cn	n	$M < F^c$	$M < F^a$	n	n	$M < F^b$
$+ + +$	n	n	$M < F^c$	n	n	n
$+ b cn$	n	$M > F^c$	$M > F^c$	NR	n	$M > F^c$
$dp + +$	n	n	n	NR	n	n
$+ + cn$	NR	NR	n	NR	NR	n
$dp b +$	NR	n	n	NR	NR	n
$+ b +$	NR	NR	n	NR	NR	NR
$dp + cn$	NR	NR	NR	NR	NR	NR
All phenotypes combined	n	$M < F^b$	$M < F^c$	n	n	n

$<$, former in minority over latter; $>$, former in majority over latter; n, non significant; NR, No recombination; M, males; d, distorted segregation; m, Mendelian segregation.
$^a P < 0.05$; $^b P < 0.01$; $^c P < 0.001$.

Distorted ($P < 0.001$) pattern of segregation was revealed in F_1 males of *D. melanogaster* from control, physical stress and 0·75% EMS experiment at dp, b and cn loci (table 3). The TC_1 males from 0·75% EMS experiment also exhibited distorted ($P < 0.001$) pattern of segregation. The TC_1 males from control and physical stress experiments, however, revealed segregation at all the 3 loci in a Mendelian fashion.

The number of males and females of *D. melanogaster* appearing in a particular phenotypic class of TC_1 and TC_2 progenies of control, physical stress and 0·75% EMS experiments were tested for 1:1 ratio. The cases revealing deviation from this sex-ratio

are mentioned. In *dp b cn* class, the females of pooled TC_1 progenies of physical stress and 0·75 % EMS experiments and in pooled TC_2 progeny of 0·75 % EMS experiments were in majority over the males ($P < 0.001$). Wildtype (+ + +) males appeared in lesser ($P < 0.001$) frequency than the females of pooled TC_1 progeny of the 0·75 % EMS experiment.

Discussion

Appearance of very high frequencies of recombination in *dp b* region in test cross progenies of TC_1 males from physical stress and 0·75 % EMS experiments suggested that induced male recombination in *D. melanogaster* was transmitted to next generation. Woodruff and Thompson (1977) reported that even after keeping a male recombination line, extracted from a natural population, for five and a half years in the laboratory, a high level of recombination was observed. Cronmiller and Cline (1983) observed that by injecting unfractionated DNA into M-strain eggs, a small recombination was observed for more than one generation. In one of the males which produced 3·7 % recombinants in F_1, recombination after increasing upto 13·2 % in F_2 of the original injected male, suddenly stopped in F_3.

The frequency of recombination, induced in the present study with physical stress and 0·75 % EMS in *dp b cn* region of *D. melanogaster* (in all the phenotypes collectively), remained statistically the same in TC_1 and TC_2 generations. Thus the enhanced male recombination caused by physical stress (with or without EMS) was transmissible.

The test cross progenies of F_1 males from all the experiments revealed the superiority of wild type over the triple recessive type and that of + *b cn* over *dp* + + type. This imbalance in 1:1 ratio of complementary products is also reflected, in all the TC_1 progenies, in the form of distorted segregation patterns at the *dp, b* and *cn* loci. In all cases where distorted segregation was observed, wild-type alleles were present in higher frequency over mutant alleles. Woodruff and Layman (1980) concluded that recombination and meiotic drive in natural populations of *D. melanogaster* were due to elements on the second chromosome region between *b* (21–48·0) and *cn* (2R −57·5), containing segregation distortion (SD) system. According to Peacock and Erickson (1965), only half of the sperm of *Drosophila* males were capable of fertilizing female eggs in the case of SD; this half included mainly those gametes that carried wild-type second chromosome. SD observed with physical stress and 0·75 % EMS in the present study may be the result of meiotic drive caused by effect(s) on the SD system.

The distorted segregation patterns observed in the TC_1 progenies of 0·75 % EMS-treated F_1 males were transmitted to next generation whereas the distorted segregation patterns exhibited by F_1 males from control and physical stress experiments were not. This may be attributed to the chemical, EMS.

The present data suggested that a major portion of recombinants detected in TC_1 and TC_2 progenies of F_1 and TC_1 males of *D. melanogaster* from physical stress and 0·75 % EMS experiments appeared in clusters. For example, one cluster in TC_1 progeny of one 0·75 % EMS-treated F_1 male comprised of as many as 28 (19 males and 9 females) + *b cn* recombinants. These clusters might have lead to the appearance of two complementary products (+ *b cn* and *dp* + +) in unequal frequency. Furthermore,

clusters of recombinants induced with physical stress and 0·75 % EMS showed non-poisson distribution. Clustering of recombinants may be attributed to variable gonial multiplication of products of occasional cross overs as suggested by Whittinghill (1947), Whittinghill and Lewis (1961), Meyer (1952), Mickey (1963) and Sharma and Swaminathan (1968). In the present study, the F_1 larvae were treated wtih 0·75 % EMS in the middle one-third part of larval life. During this period, spermatogonia are predominantly present in larval testis (Miglani and Thapar, 1983c). Majority of the recombinants recovered seem to be of premeiotic origin.

Coefficient of dispersion values indicated that the recombination events were clumped (contagious). This suggested that occurrence of one recombination event enhanced the probability of second such event (Sokal and Rohlf, 1969).

Since *D. melanogaster* has XX-XY type of sex-determination, random union between eggs and sperm at fertilization should result approximately equal numbers in XX (female) and XY (male) zygotes. In certain cases, sex-ratio imbalance was noted in the present study. In pooled TC_1 progeny (all phenotypes combined) of physical stress experiment, sex-ratio imbalance vanished in the next (TC_2) generation but that exhibited in pooled TC_1 progeny of 0·75 % EMS experiment, however, revealed itself again in TC_2 generation; overall, females predominated over males. Considering various phenotypes separately, wherever sex-ratio imbalance was significant, in the parental (+ + + and *dp b cn*) phenotypes, females in all the experiments predominated over males and in + *b cn* class, males in such cases predominated over females, in both TC_1 and TC_2 progenies of *D. melanogaster*. This suggested that male recombinant gametes carrying Y-chromosome are playing greater role in final realization of the recombinant adults as compared to those carrying X-chromosome.

The data suggest possible association of non-reciprocal male recombination, segregation distortion and sex-ratio imbalance; in fact these may be representing different aspects of the same phenomenon.

Acknowledgement

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Short Communication

N-[2-Naphthyl]-glycine hydrazide, a potent inhibitor of DNA-dependent RNA polymerase of *Mycobacterium tuberculosis* $H_{37}R_v$

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Abstract. N-[2-Naphthyl]-glycine hydrazide has been shown for the first time as a potent inhibitor of the DNA-dependent RNA polymerase (EC 2.7.7.6) of *Mycobacterium tuberculosis* $H_{37}R_v$. At a concentration of 10^{-9} M, the compound shows maximum inhibition of the enzyme, the inhibition being less at higher concentrations. It is suggested that the novel type of inhibition pattern may be due to hydrophobic interactions occurring between the molecules of the compound at higher concentrations. The finding that there is a shift in the λ_{max} of the compound could also account for this phenomenon. The effect of this compound was also tested on DNA-dependent RNA polymerases from an eukaryotic fungus, *Microsporum canis*. At a concentration of 10^{-9} M it inhibits RNA polymerase II (32%) but not RNA polymerases I and III.

Keywords. N-[2-Naphthyl]-glycine hydrazide; *Mycobacterium tuberculosis*; *Microsporum canis*; RNA polymerase.

Introduction

The DNA-dependent RNA polymerase from *Mycobacterium tuberculosis* $H_{37}R_v$ has been purified to homogeneity (Harshey and Ramakrishnan, 1976a) and it is about 1000 times more sensitive to rifampicin than the enzyme from *Escherichia coli*. Thus the polymerase of *M. tuberculosis* appears to be an ideal target for chemotherapy. In the present communication, we wish to report that the compound N-[2-naphthyl]-glycine hydrazide (compound 1415)
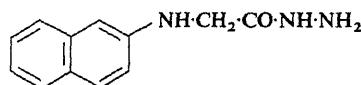
which inhibits the growth of *M. tuberculosis* $H_{37}R_v$ (Ramamurthy *et al.*, 1978), acts as a potent inhibitor of the DNA-dependent RNA polymerase.

Materials and methods

Enzymes

M. tuberculosis $H_{37}R_v$ DNA-dependent RNA polymerase was prepared according to Harshey and Ramakrishnan (1976a). DNA-dependent RNA polymerases I, II and III were purified to homogeneity from the fungus *Microsporum canis* (Rekha Prabhu, G. Ramananda Rao and T. Ramakrishnan, unpublished data).

Substrates

The sodium salts of ATP, GTP, CTP and UTP were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. [^{3}H]-UTP (13·6 Ci/mmol) was purchased from Amersham, England. Calf thymus DNA, DTT, Tris and EDTA were purchased from Sigma Chemical Co. All other reagents were of highest grade commercially available. N-[2-Naphthyl]-glycine hydrazide was prepared by the method of Tien *et al.* (1958).

Assay

The DNA directed incorporation of [^{3}H]-UMP into cold acid insoluble precipitate catalysed by RNA polymerase of *M. tuberculosis* and *M. canis* was measured. RNA polymerase assay system is as given in legend to table 1. The amount of protein was estimated according to Lowry *et al.* (1951), using crystalline serum albumin as standard.

Results and discussion

Various concentrations of the compound starting from 10^{-4} M to 10^{-11} M were prepared and tested for the inhibitory action on the mycobacterial RNA polymerase. The maximum inhibition of 80% was brought about by the compound at a concentration of 10^{-9} M (table 1). At a higher concentration the percentage of inhibition decreased. In an attempt to find out the reason for the decrease in inhibition at higher concentrations, the ultra-violet spectrum of the compound was taken at different concentrations (figure 1). The compound shows a λ_{max} at 242 nm. The spectrum shows a shift of λ_{max} by 1–2 nm at 10^{-7} M of the compound. This may be due to the hydrophobic interactions between molecules of N-[2-naphthyl]-glycine hydrazide. The effect of the compound on the 3 RNA polymerases from *M. canis*, an eukaryotic organism was also studied. RNA polymerases I, II and III from *M. canis* used in this study were purified by 25, 80 and 50-fold respectively and characterised, based on their sensitivity to mushroom toxin α-amanitin. At a concentration of 10^{-9} M the compound had no effect on RNA polymerases I and III, but only partially inhibited RNA polymerase II by 32%.

The studies with N-[2-naphthyl]-glycine hydrazide demonstrate that the compound acts as a potent inhibitor of the DNA-dependent RNA polymerase of *M. tuberculosis* $H_{37}R_v$, showing no effect on two RNA polymerases from *M. canis*. The compound has no effect on the DNA-dependent DNA polymerase of *M. tuberculosis*. If the site of action of the compound is at the level of DNA, the compound would have normally inhibited the enzyme DNA polymerase. It is therefore plausible that the compound is

Table 1. The inhibition of RNA polymerase activity from *M. tuberculosis*.

Concn. of inhibitor	Inhibition of the activity of RNA polymerase (%)
10^{-4} M	40
10^{-5} M	59
10^{-6} M	48
10^{-7} M	50
10^{-9} M	80
10^{-10} M	58
10^{-11} M	53

The assay mixture contained in a final volume of 0·1 ml: Tris HCl (pH 7·8), 0·1 M; $MgCl_2 \cdot 6H_2O$, 6 mM; $MnCl_2$, 2 mM; $(NH_4)_2SO_4$, 0·05 M; DTT, 4 mM; XTPs, 1 mM; UTP, 0·01 mM; calf thymus DNA (native and denatured), 10 μg; [^{3}H]-UTP, 0·2 μCi, inhibitor N-[2-naphthyl]-glycine hydrazide at varying concentrations. The reaction was started by adding the enzyme (0·2 mg of protein) and carried out at 30°C for 10 min. The activity in controls (100 %) corresponds to incorporation of 88 pmol of [^{3}H]-UMP.
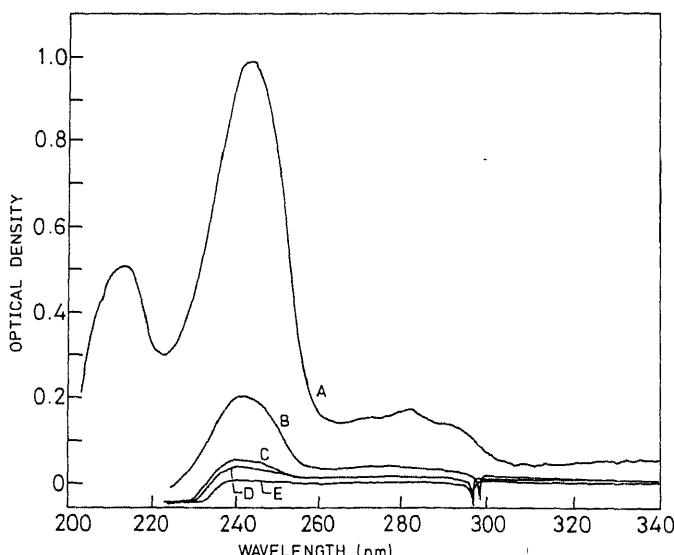
Figure 1. The ultra-violet spectrum of N-[2-naphthyl]-glycine hydrazide at concentrations of 10^{-5} M (A), 10^{-6} M (B), 10^{-7} M (C), 10^{-8} M (D) and 10^{-9} M (E) in aqueous solutions adjusted to pH 7.

directly acting on RNA polymerase and not on DNA. The finding that 100% inhibition of the enzyme activity is not achieved even at higher concentrations could be due to the presence of two forms of the enzyme, *viz.*, H and L forms in *M. tuberculosis* (Harshey and Ramakrishnan, 1976b). The action of the inhibitor on these two forms could be different, which could also account for the anomalous pattern of inhibition observed. Further, the compound at low concentrations could have an effect on the ρ component of the polymerase thereby bringing about chain termination, but at higher concentrations the compound could act on the subunit and thereby promoting transcription, this in turn resulting in lower inhibition at higher concentrations. A similar observation, *viz.*, that the inhibition of RNA polymerase of *E. coli* by myxopyrenin was never complete even at very high concentrations has been reported (Irschik *et al.*, 1985). The fact that N-[2-naphthyl]-glycine hydrazide inhibits specifically the DNA-dependent RNA polymerase of *M. tuberculosis* with insignificant inhibition of the eukaryotic enzyme, and that it is non-mutagenic (Ramamurthy *et al.*, 1985) makes the compound a potential drug for the treatment of tuberculosis in man.

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Short Communication

Antifertility effect of tamoxifen as tested in the female bonnet monkey (*Macaca radiata*)

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Abstract. The administration of a potent antiestrogen, tamoxifen at a dose of 3 mg/kg body weight/day orally post-coitally to cycling mated bonnet monkeys (*Macaca radiata*) from days 18–30 of cycle resulted in inhibition of establishment of pregnancy in 9 out of 10 monkeys. Tamoxifen effect was not due to interference with luteal function. The effect was specific to tamoxifen as exogenously administered progesterone could not reverse it. In addition to suggesting a role for estrogen in maintenance of early pregnancy in the primate the present study could be a prelude to the development of an effective post-ovulatory approach for regulation of fertility in the human female.

Keywords. Antiestrogen; implantation; pregnancy; primate; post-coital contraception.

Introduction

The need for estrogen for maintenance of early pregnancy in the primate is presently questioned. The idea that estrogen probably is not required for implantation and immediate postimplantation survival of the blastocyst in primates (possibly including the human) stems from the observation that monkeys ovariectomized within 4–5 days of mating or luteectomy on day 6 post-coitum will continue with pregnancy if supplemented with progesterone alone (Meyer *et al.*, 1969; Bosu and Johansson, 1975). A variety of antiestrogens which are effective in inhibiting implantation in the rat are ineffective when administered post-coitally in the monkey (Morris *et al.*, 1967). However, most of the compounds used thus far—clomiphene, MER-25, centchroman and others are known to have, in addition to antagonistic, pronounced estrogenic activity. Further, as the time of administration of these drugs does not overrule the effect they may have on tubal motility, blastocyst survival etc., the need of estrogen for implantation *per se* and postimplantation survival of the blastocyst remains to be convincingly demonstrated (Prasad, 1985). There is a suggestion that in the primate the preovulatory estrogen surge in itself may be adequate to sensitize the uterus for implantation to occur (Prasad, 1985). In the context of present world-wide interest in

the use of antiprogestins to terminate early pregnancy and the lack of clarity regarding the need for estrogen in maintaining early pregnancy in the primate, it was decided to study the effect of administration of a relatively pure antiestrogen such as tamoxifen in mated bonnet monkeys, starting from mid luteal phase, on the establishment of pregnancy. Tamoxifen [(Z)-2-{4-(1,2-diphenylbut-1-enyl) phenoxy}-N, N-dimethyl-ethanamine] (gift of Dr B. J. A. Furr, I.C.I., UK) is an antiestrogenic compound having apparently no estrogenic activity as tested in the pig tailed monkey and the human (Furr *et al.*, 1979). It is presently primarily used as a therapeutic agent in controlling estrogen dependent breast cancer (Furr *et al.*, 1979).

Methods

A group of 32 cycling bonnet monkeys with a proven history of normal cycles and pregnancies were mated between days 9–14 of cycle with proven fertile males. Information regarding the husbandry of bonnet monkeys and their gonadal hormone profiles during the cycle and early pregnancy have been reported earlier (Moudgal *et al.*, 1978). In particular, the ovulatory nature of cycles during the treatment phase was adjudged by monitoring serum estrogen levels between days 7–10 and progesterone levels between days 16–18 of the cycle. Since these monkeys ovulate between days 11–12 of cycle, fertilization should have occurred between days 11–13 of cycle. The first group of 6 monkeys (control group) did not receive any treatment. The second group of 10 monkeys were treated with tamoxifen orally, 3 mg/kg body weight daily from day 18 of cycle for 13 days. The third group of 16 monkeys also received tamoxifen between days 18–30; however, 5 of them were supplemented with intramuscular injections of 10 mg of medroxyprogesterone acetate (MPA) while 11 were given 10 mg progesterone/monkey/day from day 18–30. By providing a gap of 4–7 days between fertilization and start of tamoxifen therapy we have ensured that its effect is confined to events beyond tubal development of the fertilized ovum. Blood samples collected by femoral venipuncture at regular intervals till the termination of cycle were assayed for serum estradiol, progesterone and monkey chorionic gonadotropin (mCG) by appropriate radioimmunoassays standardised in this laboratory (Rao *et al.*, 1984). Serum samples collected between days 28–35 of cycle, from those mated monkeys which did not show premature break of cycle, were used for mCG assay. The present study was undertaken during the months of August–December when the animals are most fertile.

Results and discussion

It is evident from table 1 that tamoxifen is effective in the inhibition of initiation/establishment of pregnancy in 22 out of 26 monkeys. All the 32 monkeys used in the present study were proven fertile females. Based on the colony performance for the last 4 years and from the data included for controls in the present study, 60–70 % of the monkeys conceive and go through pregnancy when exposure to the male is restricted to one cycle only.

Out of 6 monkeys in the control group (group I), 4 became pregnant and continued to term.

Table 1. Effect of tamoxifen administration during postcoital phase on conception in bonnet monkeys.

Group	Treatment[a]	Number pregnant/ Number mated	Peak estradiol (pg/ml) levels during follicular phase (mean ± SEM)	Progesterone (ng/ml) levels on day 18 of cycle (mean ± SEM)	pregnant (%)	protection[c] (%)
I	Nil	4/6	388 ± 74·9	5·00 ± 2.8	66	—
II	Tamoxifen	1/10	388 ± 58·4	6·28 ± 0·9	10	84
III	Tamoxifen + progestin[b]	3/16	720 ± 120·9[d]	3·43 ± 0·6	18	70

[a] All monkeys were mated with proven fertile males between days 9–14 of cycle. Excepting those in group I the rest of the monkeys were administered tamoxifen at 3 mg/kg body weight per monkey per day from days 18–30 of cycle.

[b] In the group III, 11 monkeys received progesterone at 2 mg/kg body weight per monkey per day and 5 monkeys received MPA at 2 mg/kg body weight per monkey per day from days 18–30 of cycle.

[c] Per cent protection was calculated assuming that even in the Experimentals % conception in the absence of treatment would have been 66% like in controls.

[d] Five animals showed values ranging from 800–1200 pg/ml while 11 monkeys showed values ranging from 250–380 pg/ml.

In group II fed tamoxifen alone, out of 10 monkeys, one became pregnant and continued to term; 5 monkeys bled prematurely on day 24 itself and of the other 4 monkeys which were mCG positive on day 28 of cycle, 3 bled on day 29 and one on day 56. Even in the monkey that bled on day 56, pregnancy termination must have occurred much earlier as no fetal remnants could be recovered in the menstrual blood; this is to be expected if the monkey had aborted this late in pregnancy due to some other reason. The present observation that tamoxifen can block conception to the extent of 84% over the control is statistically significant at 1% level according to the test of significance.

The specificity of the effect of tamoxifen is evident from the fact that neither progesterone nor MPA, a long acting progestin, could reverse the effect significantly. Out of 16 animals supplemented with progestin, 3 continued with pregnancy, indicating a drop in percentage protection from 84% to 70% over the control value. But the overall protection rate was 85% considering both tamoxifen and tamoxifen + progestin treated groups.

The observation that 5 of the tamoxifen treated monkeys showed premature bleeding (earlier than day 28) is of significance and may imply an interference with the implantation process *per se*. In the tamoxifen + progestin supplemented group only one showed such an effect and this is perhaps due to the protective effect of the supplemented progestin against endometrial bleeding. In our experience administration of tamoxifen at 3 mg/kg dose to cycling but nonmated monkeys results in an extension of the cycle length from a normal 28 ± 2 to 48·6 ± 3·9 days. Following tamoxifen treatment, the luteal phase of the cycle, based on the progesterone levels was not curtailed but extended from a normal of 18 ± 2·1 to 38·6 ± 4·0 days.

The present study thus provides direct evidence for the involvement of estrogen in maintenance of early pregnancy in the primate. Since tamoxifen exerts its effect even in the presence of normal serum estrogen titers, it is perhaps acting by blocking uterine

estrogen receptors and also interfering with the turnover of both estrogen and progesterone receptors. The observation that tamoxifen administered during the post-ovulatory phase is effective in blocking implantation indicates that the low level of estrogen present during the early luteal phase may have a role in the initiation of implantation. Since the half-life of tamoxifen is long ($\sim$ 7 days) (Furr *et al.*, 1979), it may be possible to reduce the dosage and duration of administration without impairing its contraceptive efficacy. The per cent protection against conception can perhaps be further increased by initiating the treatment on day 16 of the cycle itself. Currently experiments are underway to explore this possibility. It may also be of interest to note that monkeys treated with tamoxifen are capable of achieving normal conception in subsequent cycles. This study may portend the use of an antiestrogen as an effective post-ovulatory, once-a-month contraceptive agent for regulation of fertility in the human female.

Acknowledgements

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Lipoic acid and diabetes—Part III: Metabolic role of acetyl dihydrolipoic acid

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Abstract. Rat liver lipoyl transacetylase catalyzes the formation of acetyl dihydrolipoic acid from acetyl coenzyme A and dihydrolipoic acid. In an earlier paper the formation of acetyl dihydrolipoic from pyruvate and dihydrolipoic acid catalyzed by pyruvate dehydrogenase has been reported. Acetyl dihydrolipoic acid is a substrate for citrate synthase, acetyl coenzyme A carboxylase and fatty acid synthetase. The V_{max} for citrate synthase with acetyl dihydrolipoic acid was identical to acetyl coenzyme A (approximately 1 μmol citrate formed/min/mg protein) while the apparent K_m was approximately 4 times higher with acetyl dihydrolipoic acid as the substrate. This may be due to the fact that synthetic acetyl dihydrolipoic acid is a mixture of 4 possible isomers and only one of them may be the substrate for the enzymatic reaction. While dihydrolipoic acid can replace coenzyme A in the activation of succinate catalyzed by succinyl coenzyme A synthetase, the transfer of coenzyme A between succinate and acetoacetyl dihydrolipoic acid catalyzed by succinyl coenzyme A: 3 oxo-acid coenzyme A transferase does not occur.

Keywords. Lipoic acid; acetyl coenzyme A; acetyl dihydrolipoic acid; citrate synthase.

Introduction

In an earlier study (Natraj *et al.*, 1984) we have shown that lipoic acid administration in alloxan diabetic rats alleviated many of the biochemical abnormalities in diabetes. Major impairments of carbohydrate, fat and protein metabolisms occur due to reduced uptake and utilization of glucose in diabetes. Adequate intracellular concentrations of glucose are met through its supply from liver and kidney where it is synthesised from precursors such as alanine, lactate, etc. resulting in increased gluconeogenic rates (Hers and Hue, 1983). The energy requirements in the liver and kidney are primarily met through fatty acid oxidation resulting in elevated levels of acetyl coenzyme A (CoA), acetoacetate and β-hydroxy butyrate (Randle, 1966). Higher levels of acetyl CoA in diabetes are believed to be responsible for the inhibition of pyruvate dehydrogenase (EC 1.2.4.1) (Kerbey *et al.*, 1976) and activation of pyruvate carboxylase (EC 6.4.1.1) (Williamson *et al.*, 1969; Ruderman *et al.*, 1976; Utter *et al.*, 1964). From this point of view, reduction in acetyl CoA levels would therefore be expected to have a beneficial effect in diabetes.

Abbreviations used: CoA, Coenzyme A; DTO, dihydrolipoic acid; DTNB, 5, 5 dithiobis (2-nitrobenzoic acid); BSA, bovine serum albumin; DTT, dithiothreitol; NAD, nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; TLC, thin layer chromatography.

Like CoA, dihydrolipoic acid (DTO) possesses a sulphydryl group and can form thiol esters. In the present study we have shown that acetyl DTO was formed from acetyl CoA and DTO thus leading to a net reduction in acetyl CoA levels. Furthermore, acetyl DTO replaced acetyl CoA in acetyl CoA carboxylase (EC 6.4.1.2) and fatty acid synthetase catalyzed reactions which are key to fatty acid biosynthesis. Similarly, DTO was found to replace CoA in succinyl CoA synthetase (EC 6.2.1.4) but not in succinyl CoA: 3 oxo-acid CoA transferase (EC 2.8.3.5). Thus thiol esters of DTO performed some of the functions of CoA thioesters and not others. The implications of this selective role of DTO in bringing about the changes in diabetes have been discussed.

Materials and methods

D,L-Lipoic acid was purchased from British Drug House, England. [^{14}C]-Bicarbonate, and [1-^{14}C]-acetic anhydride were purchased from Bhabha Atomic Research Centre, Bombay. Bovine serum albumin (BSA), CoA, dithiothreitol (DTT), 5,5 dithiobis (2-nitrobenzoic acid) (DTNB), malonyl CoA, nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), oxalacetate and sodium pyruvate were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. All other reagents were of analytical grade. The rats used were an inbred Haffkine Wistar strain maintained on Hindustan Lever pellet feed.

Acetyl DTO was prepared according to Gunsalus *et al.* (1956). Radioactively labelled acetyl CoA and acetyl DTO were prepared according to the procedure of Simon and Shemin (1953). For malonyl DTO the procedure described for malonyl CoA by Trams and Brady (1960) was modified as follows. Thiophenyl malonate was made from malonic acid and thiophenol, and reacted with DTO in bicarbonate buffer (0·1 M, pH 8·5). Chloroform extract of the acidified reaction mixture showed 4 spots on thin layer chromatography (TLC) in chloroform : acetone : formic acid (9 : 1 : 0·1). The spot with an R_f of 0·5 was identified as malonyl DTO by the hydroxamate method (Lipmann and Tuttle, 1945). The other bands corresponded to mixed malonate esters with thiophenol and DTO.

Acylation of DTO catalyzed by lipoyl transacetylase

The procedure described by Brady and Stadman (1954) for isolation and purification of pigeon liver lipoyl transacetylase was adapted to rat liver. The enzyme was partially purified (upto 75 % ammonium sulphate saturation step). The assay mixture in a final volume of 1·5 ml contained 100 μmol of Tris buffer pH 7·5, 0·5 μmol of acetyl CoA and 10 μmol of DTO. The reaction was started by adding the enzyme (approximately 500 μg protein) and incubated at 25°C for 30 min and terminated by adding 0·2 ml of chilled 6 N HCl and extracted with benzene. The organic layer was washed with water, dried over sodium sulphate and the solvent removed under a stream of nitrogen gas. Acetyl DTO was estimated according to Lipmann and Tuttle (1945).

Citrate synthase and acetyl DTO

Citrate synthase was isolated from normal rat liver mitochondria and partially purified

as described by Shepherd and Garland (1969) with the following modifications. The mitochondrial pellet from 60 g of liver was sonicated (20 KHz for 8 min) in 80 ml of 0·1 M potassium phosphate buffer, pH 7·4 and the homogenate was subjected to ammonium sulphate fractionation. The precipitate obtained at 50–75 % saturation was dissolved in 10 ml of 0·1 M potassium phosphate buffer, pH 7·4 and dialysed against 3 litres of distilled water. The dialysate was loaded onto a DEAE-sephacel column (2 × 15 cm) and was successively washed with 1 litre each of 2 mM and 8 mM potassium phosphate buffer, pH 7·4. The column was eluted with 18 mM potassium phosphate buffer, pH 7·4 and fractions of 250 ml were collected. The 3rd and 4th fractions had all the enzyme activity and was precipitated by 50–75 % ammonium sulphate saturation, dissolved in 10 ml of buffer and dialysed against 2 litre distilled water for 24 h. The dialyzed extract was used as the source of the enzyme.

The condensation of acetyl CoA or acetyl DTO with oxalacetate results in the release of free CoA or DTO which was measured spectrophotometrically using DTNB. The assay mixture in a final volume of 2 ml contained: 200 μmol of Tris buffer, pH 8·0, 25–200 nmol of acetyl CoA or 20–150 nmol of acetyl DTO, 0·2 μmol of DTNB and 100 μl of enzyme (approximately 20 μg protein). The reference cuvette contained all the ingredients except oxalacetate. The reaction was monitored initially for 3 min for absorbance increase at 412 nm and the assay was started by adding 0·5 μmol oxalacetate in the sample cuvette only. The change in absorbance at 412 nm was monitored.

The product of the reaction was identified by estimating the citrate formed according to Stern (1957).

Carboxylation of acetyl DTO to malonyl DTO catalyzed by acetyl CoA carboxylase

Acetyl CoA carboxylase was partially purified from normal rat livers (up to 25 % ammonium sulphate saturation step) according to the procedure of Inoue and Lowenstein (1975). The enzyme was assayed by estimating the amount of $NaH^{14}CO_3$ incorporated into malonate. The assay mixture, in a final volume of 2 ml contained: 200 μmol Tris HCl buffer, pH 7·5, 2·5 μmol DTT, 0·8 μmol acetyl CoA or acetyl DTO, 40 μmol of $NaH^{14}CO_3$ (4·1 × 10^6 CPM), 10 μmol of ATP, 40 μmol of citrate, 40 μmol of NaCl and 1 mg BSA. The reaction was started by adding an aliquot of the enzyme (approx. 100 μg protein) and incubated at 37°C for 15 min. The reaction was stopped by adding 1 ml of 6 N HCl and evaporating the mixture to dryness in a hot water bath under a stream of nitrogen. The residual radioactivity was counted in a scintillation spectrometer (Packard). The control for this experiment contained all the ingredients except ATP and the radioactivity incorporated was estimated and the value was subtracted from the experimental values.

In a separate experiment, 0·8 μmol of CoA was added along with acetyl DTO and the assay performed as described above.

The product of the reaction was identified by extracting the acidified reaction mixture with chloroform and the solvent removed under a stream of nitrogen gas. The residue was separated on TLC using hexane: ether: acetic acid (80: 20: 1). The spots corresponding to acetyl DTO and malonyl DTO were eluted and estimated by conversion to hydroxamate according to the method of Lipmann and Tuttle (1945).

Acetyl DTO as a substrate for fatty acid synthetase

Fatty acid synthetase was isolated and partially purified upto the DEAE cellulose chromatography step (Stoops *et al.*, 1979) and was assayed by coupling with acetyl CoA carboxylase and measuring the rate of oxidation of NADPH. The assay mixture in a final volume of 1 ml contained: 40 μmol of Tris HCl buffer, pH 7·5, 8 μmol of MgCl$_2$, 8 μmol of sodium citrate, 3 μmol of DTT, 20 μmol of sodium bicarbonate, 0·6 mg BSA, 0·1 μmol acetyl CoA or acetyl DTO, 0·1 μmol of NADPH, acetyl CoA carboxylase (approximately 100 μg protein), and fatty acid synthetase (approximately 100 μg protein). The assay mixture was taken in the sample and reference cuvettes in a double beam spectrophotometer (Shimadzu, UV 240) and the reaction was started by adding 3 μmol of ATP to the sample cuvette only and the absorbance change at 340 nm followed spectrophotometrically. This procedure would correct for NADPH oxidase that may be present in the enzyme sample which would otherwise have given higher values for enzyme activity.

The product of the above assay was identified by substituting acetyl DTO with [1-^{14}C]-acetyl DTO. The reaction mixture was incubated for 15 min at 37°C and acidified with 1 ml of 6 N HCl. The products were extracted into chloroform and the solvent was evaporated under a stream of nitrogen gas. The fatty acids were separated by TLC using hexane:ether:acetic acid (80:20:1). The radioactivity in the spot corresponding to palmitate was determined.

Activation of succinate catalyzed by succinyl CoA synthetase

The enzyme was isolated from rat heart according to the method described by Murakami and Nishimura (1974). The enzyme was assayed by measuring the increase in absorbance at 235 nm for the succinyl thioester formed.

The assay mixture in a final volume of 1 ml contained: 50 μmol of Tris succinate buffer, pH 7·4, 10 μmol of MgCl$_2$, 0·1 μmol of thiol (CoA or DTO) and 200 μg of enzyme protein. The reaction was started by adding the enzyme and the increase in absorbance at 235 nm with time was monitored spectrophotometrically (Sungman, 1969).

Noninvolvement of succinyl DTO in succinyl CoA : 3 oxo-acid CoA transferase

The enzyme was isolated from rat hearts and purified by ammonium sulphate fractionation (35–65% saturation), acetone precipitation (57% saturation) and heat and acid treatment (Stern *et al.*, 1956). The enzyme was concentrated by ammonium sulphate precipitation (72% saturation) and dialysed. The dialysate was further purified by DEAE-sephacel column chromatography (Hersh and Jencks, 1967). The purified enzyme was free of thiolase activity.

The enzyme activity was determined by measuring the decrease in the 310 nm absorbance of acetoacetate thioester with time. The assay mixture in a final volume of 1 ml contained: 70 μmol of Tris-HCl buffer, pH 8·1, 50 μmol of succinate, 0·2 μmol of acetoacetyl thioester and 50 μg of enzyme protein. The hydrolysis of acetoacetyl thioester was followed for 2–3 min and the reaction was started by the addition of succinate. One unit of enzyme activity is defined as an absorbance change of 0·01/min at 310 nm which is equivalent to 2·5 $\times$ 10^{-3} μmol of acetoacetyl CoA or acetoacetyl DTO.

Results

Formation of acetyl DTO

The assay of lipoyl transacetylase described by Brady and Stadman (1954) used a system generating acetyl CoA from acetyl phosphate and CoA. Formation of acetyl CoA was monitored by taking aliquots of the assay mixture and estimating the thioester content by the hydroxamate assay. In order to demonstrate that excess acetyl CoA in the liver can be transacylated to acetyl DTO, the former was used in the present study as the acyl donor. The enzyme activity was measured by separating acetyl DTO from acetyl CoA by making use of their differential solubility in benzene and estimating the thioester by conversion to hydroxamate. The results showed that rat liver lipoyl transacetylase catalyzed the conversion of acetyl CoA to acetyl DTO at 90 nmol/min/mg protein in the presence of excess DTO.

Acetyl DTO as a substrate for citrate synthase

Crude citrate synthase contains an active hydrolase which hydrolyses acetyl DTO at appreciable rates. Purified citrate synthase was virtually free of any hydrolase activity as evidenced by negligible DTO formation in the absence of any oxalacetate. The results of citrate synthase assay are shown in figure 1. In the presence of acetyl DTO it was found that there was a rapid release of free –SH groups as measured by the reaction with DTNB. The K_m value for acetyl CoA was estimated to be 14 μM. With acetyl DTO as the substrate, the V_{max} was identical to that of acetyl CoA but the K_m value was found to be 62 μM which was approximately 4 times larger than that for acetyl CoA. The product of the reaction was identified as citrate by the colorimetric method described by Stern (1957).

Carboxylation of acetyl DTO catalyzed by acetyl CoA carboxylase

The results demonstrated that acetyl DTO served as a substrate for acetyl CoA carboxylase and was converted to malonyl DTO. Specific activity of the enzyme with acetyl DTO as a substrate (44·7 nmol of HCO_3^- fixed/min/mg protein) was nearly 60 % of that with acetyl CoA (74·9 nmol of HCO_3^- fixed/min/mg protein). The velocities observed with and without added CoA were similar suggesting that acetyl DTO was not converted first to acetyl CoA and then to malonyl DTO. This indicates that acetyl CoA is unlikely to be the intermediate for the carboxylation of acetyl DTO.

Acetyl DTO as a substrate for fatty acid synthetase

Fatty acid synthetase catalyzes the successive condensation of 7 malonyl CoA molecules with acetyl CoA to produce palmitate. In the present study malonyl-CoA or malonyl-DTO was produced *in situ* from acetyl CoA or acetyl DTO catalyzed by acetyl CoA carboxylase. This reaction was coupled to fatty acid synthetase and the overall rate was monitored through oxidation of NADPH. In a separate assay, [1-^{14}C]-acetyl CoA and [1-^{14}C]-acetyl DTO were used as substrates and the radioactivity incorporated into palmitate was quantified.

From table 1 it can be seen that acetyl DTO was converted to palmitate with the
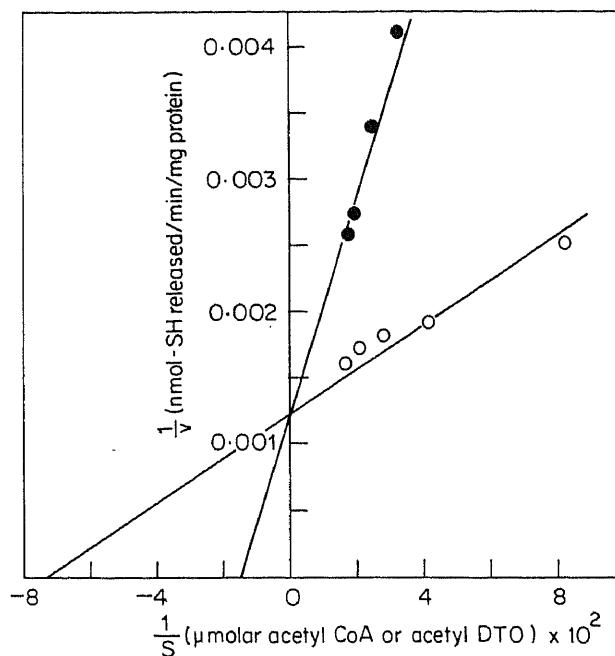
Figure 1. Effect of varying concentrations of acetyl CoA (O) and acetyl DTO (●) on citrate synthase activity. The assay mixture in a volume of 2 ml contained: 200 μmol Tris buffer, pH 8·0, 0·5 μmol oxalacetate, 25–200 nmol of acetyl CoA or 20–150 nmol of acetyl DTO and 0·2 μmol DTNB. The reaction was started by adding an aliquot of citrate synthase (approximately 20 μg protein) and recording the absorbance change at 412 nm. The results analysed by Lineweaver-Burk plot.

Table 1. Acetyl DTO as a precursor for palmitate.

	Activity	
Substrate	nmol NADPH oxidized/ min/mg/protein	nmol [^{14}C]-acetyl CoA/DTO incorporated/min/mg/protein
Acetyl CoA	75·0 (5·6)	43·2 (5·4)
Acetyl DTO	37·0 (2·7)	22·2 (2·7)

Fatty acid synthetase was isolated from normal rat livers and the activity estimated by coupling with acetyl CoA carboxylase and determining the rate of oxidation of NADPH (nmol/min/mg protein) or, the radioactivity incorporated from [1-^{14}C]-acetyl CoA or acetyl DTO into palmitate (nmol/min/mg protein). The numbers in parentheses are the nmol of palmitate produced/min/mg protein.

relative rate approximately 50% of that obtained with acetyl CoA both by the spectrophotometric and radioactivity assays. After correcting for losses during recovery of palmitate in the radioactive assay, the rate of palmitate formation (numbers in parentheses in table 1) was identical in both assays.

Succinyl CoA synthetase

Succinyl CoA synthetase catalyzes the activation of dicarboxylic acids with CoA in the presence of GTP. As observed with citrate synthase and acetyl CoA carboxylase, DTO substituted for CoA, although the enzyme activity with DTO as the substrate was approximately 40% of that with CoA (10 and 4 nmol of thioester formed/min/mg protein with CoA and DTO respectively).

Succinyl DTO does not participate in succinyl CoA : 3 oxo-acid CoA transferase

The enzyme isolated from rat heart was purified to eliminate the risk of any thiolase contamination which would otherwise have interfered with the assay. The results of the thiol transfer from acetoacetyl CoA or acetoacetyl DTO to succinate are shown in table 2. The enzyme catalyzed the transfer of CoA between acetoacetyl CoA and succinate but not between acetoacetyl DTO and succinate indicating that the enzyme had a high degree of specificity for the thiol moiety.

Table 2. Transfer of DTO catalyzed by succinyl CoA : 3 oxo-acid CoA transferase.

| | Activity | |
| | Substrate converted | |
Substrate	A_{310}/min/mg protein	μmol/min/mg protein
Acetoacetyl CoA	70·6	0·176
Acetoacetyl DTO	ND	ND

ND, Not detectable.
The enzyme was isolated from normal rat heart and purified to remove any contaminating thiolase activity. The enzyme was assayed by following the decrease in 310 nm absorbance.

Discussion

In an earlier study (Gandhi *et al.*, 1985) the mode of action of lipoic acid in diabetes was shown to be through its effects on certain enzymes of fatty acid oxidation and gluconeogenesis, which were slowed down, thus accounting for a reduction in ketosis and gluconeogenesis. It was shown that DTO replaced CoA effectively in the oxidative decarboxylation of pyruvate catalyzed by pyruvate dehydrogenase to form acetyl DTO. DTO was found to activate long chain fatty acids as DTO thioesters catalysed by fatty acyl CoA synthetase (EC 6.2.1.3). Two important differences between CoA and DTO observed were that (i) acetoacetyl DTO was a poor substrate for thiolase (EC 2.3.1.9)

while acetoacetyl thioesters of pantetheine and N-acetyl β-mercaptoethylamine have been reported to be good substrates (Stern and Drummond, 1961) and (ii) acetyl DTO did not activate pyruvate carboxylase to the same extent as acetyl CoA. It appeared therefore that DTO had a specific role and was not a nonspecific sulphydryl compound, whose only resemblance to CoA was in its thiol group(s); such a specific role is most likely to be in fatty acid biosynthesis. We have presented some evidence to substantiate this hypothesis.

In the present study, we have examined the origin and the metabolic fate of acetyl DTO. Lipoyl transacetylase has been isolated from rat liver and shown to catalyze the formation of acetyl DTO from acetyl CoA and DTO. Acetyl DTO has been shown to be a substrate for citrate synthase, acetyl CoA carboxylase and fatty acid synthetase, 3 enzymes which catalyze crucial steps in the synthesis of palmitate. Thus DTO by substituting for CoA in these reactions may be expected to support fatty acid biosynthesis under the conditions where CoA availability may become limiting as has been suggested in diabetes (Sauer and Erfle, 1966), or even in normal conditions as an alternate pathway for fatty acid biosynthesis.

The role of DTO in relation to the metabolic fate of acetoacetate has also been investigated. DTO has been shown to replace CoA in the activation of succinate catalyzed by succinyl CoA synthetase. However, the DTO moiety in acetoacetyl DTO is not transferred to succinate in the reaction catalyzed by succinyl CoA : 3 oxo-acid CoA transferase. It must be mentioned that Moore and Jencks (1982) have shown that CoA transfer from acetoacetyl CoA to succinate is highly specific for the nonreacting portions of the CoA molecule; neither N-acetyl β-mercaptoethylamine nor pantotheine can replace CoA in this reaction.

An important difference between acetyl CoA and acetyl DTO in all these reactions is that, for a given concentration of acetyl CoA/acetyl DTO, the enzymatic reaction in the presence of acetyl CoA was faster than that with acetyl DTO. This is indicated from studies with citrate synthase which showed that the V_{max} for the two substrates was similar, but the K_m for acetyl DTO was approximately four times of that for CoA. It must be pointed out that synthetic acetyl DTO is a mixture of 4 possible isomers: (+)8-S-acetyl, (−)8-S-acetyl, (+)6-S-acetyl and (−)6-S-acetyl dihydrolipoic acid. It is probable that only one of these is the substrate with the result that the actual concentration of the active compound is considerably lower than the total (one fourth?). Support for this interpretation also stems from observations by Gunsalus *et al.* (1956) who showed that the product of the enzymatic acylation of exogenous DTO by acetyl CoA catalyzed by extracts of *Escherichia coli* was the 6-S-acetyl derivative. Studies by O'Connor *et al.* (1982) with *E. coli* and bovine kidney pyruvate dehydrogenase showed that the principal product was the 8-S-acetyl derivative. It is probable that this specificity may also be found in the other enzyme reactions we have examined. At present the chemical identity of the acetyl derivative is not known and work is in progress to clarify this issue.

These investigations clearly demonstrate the need to re-examine the role of lipoic acid in intermediary metabolism. While its function as a coenzyme in the enzyme complexes that catalyze the oxidative decarboxylation of α-keto acids is well established, the findings from this and the previous study (Gandhi *et al.*, 1985) open up the possibility that DTO may substitute for CoA in other enzyme reactions in

intermediary metabolism. The question that remains to be answered is whether infact DTO is of physiological importance in some of these metabolic functions under normal physiological conditions in the animal and if so, do DTO thioesters actually participate in fatty acid biosynthesis? Answers to this question form the theme of the experiments in progress in our laboratory.

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Fermentation pattern of *Zymomonas mobilis* strains on different substrates—a comparative study

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Abstract. The optimum conditions (pH and initial sugar concentration) of fermentation for the production of ethanol by 4 strains of *Zymomonas mobilis* (ATCC 10988, ATCC 12526, NRRL B 4286 and IFO 13756) were studied. An initial sugar concentration of 15 % (w/v) at pH 7·0 was found to be optimal for the first two strains and 20 % (w/v) initial sugar at pH 7·0 was found to be optimal for the last two strains. The fermentation pattern of these strains on synthetic medium, cane juice and molasses were compared. Strain NRRL B 4286 showed maximum ethanol production on synthetic medium while on cane juice ATCC 10988 and ATCC 12526 performed well. However, all the strains fermented molasses poorly.

Keywords. *Zymomonas mobilis*; optimization of condition; fermentation; synthetic medium; cane juice; molasses.

Introduction

Zymomonas mobilis is a rod shaped, gram negative, non-spore forming, motile bacteria. As an alternative organism for the production of ethanol, *Z. mobilis* has several advantages over yeast. These are: higher rates of glucose uptake and ethanol production, higher ethanol yields and ethanol tolerance (Lee *et al.*, 1979; Rogers *et al.*, 1979). This bacterium uses a modified Entner-Doudroff pathway to rapidly produce upto 1·9 mol of ethanol per mol of glucose used. Thus *Z. mobilis* is a promising candidate for large scale production of ethanol (Gibbs *et al.*, 1951, 1954; Swings and DeLey, 1977). In this paper we report optimization experiments (pH and initial sugar concentration) for the production of ethanol by strains of *Z. mobilis* and compared the fermentation pattern of these strains on various substrates.

Materials and methods

Strains of *Z. mobilis* were obtained from Oak Ridge National Lab, Tennesse, USA (ATCC 10988 and ATCC 12526), Northern Regional Lab, Illinois, USA (NRRL B 4286) and Institute for Fermentation, Osaka, Japan (IFO 13756). These cultures were maintained as stab cultures in YPS agar medium (yeast extract 1 %; peptone 1 %; sucrose 2 % and agar 2 %) at 4°C.

The synthetic fermentation medium contained sucrose 20 % (w/v), yeast extract 0·5 % (w/v), ammonium sulphate 0·1 % (w/v), potassium dihydrogen ortho phosphate 0·1 %

(w/v) and magnesium sulphate 0·05 % (w/v). Cane juice was used as such. Generally, the reducing sugar concentration in the cane juice was 18 % (w/v). The molasses medium was prepared by diluting the molasses as required. The pH of all these media was adjusted as required and then fermentation media were sterilized at 10 pounds for 30 min. For batch fermentation, the primary inoculum was prepared in YPS broth and incubated at room temperature for 18 h. Then the fermentation broth was inoculated with 10 % (v/v) of seed culture and fermentation process carried out at 30°C.

Total sugar was estimated colorimetrically as described by Dubois *et al.* (1956). Ethanol was estimated by gas chromatography (Model 5830A, Hewlett Packard, USA) according to Robinson *et al.* (1980) with the following modifications. Six feet 10 % carbowax 20 m column was maintained at 80°C for 2 min and increased to 140°C at the rate of 25°C per min and held for 5 min. Injector temperature was 150°C and detector temperature was 160°C. Nitrogen gas was used as carrier at 25 ml/min and ethanol as standard.

Results and discussion

Effect of initial pH on fermentation

In order to find out the optimum pH for the fermentation with *Z. mobilis* the initial pH of the fermentation medium was adjusted to different levels and fermentation carried out for 72 h. At the end of this period samples were collected and ethanol and residual sugar concentration were estimated. Table 1 shows the effect of initial pH on the fermentation parameters of various strains of *Z. mobilis*. In all, the fermentation efficiency was maximum at pH 7·0 but low at pH 4·0. With the increase of initial pH, the substrate utilization and fermentation efficiency increased. Hence, the optimum initial pH for fermentation by *Z. mobilis* is 7·0. This result is consistant with the result of Ohta *et al.* (1980).

Effect of initial sugar concentration on ethanol production

To find out optimum sugar level for fermentation, batch fermentation was carried out with varying levels of sugar. Table 2 shows ethanol produced by 4 strains of *Z. mobilis* in varying initial sugar concentration. The maximum efficiency of fermentation was observed at 15 % (w/v) initial sugar concentration for strain ATCC 10988 and ATCC 12526. Increasing the sugar concentration from 15–20 % decreased the fermentation efficiency of these strains. However IFO 13756 and NRRL B 4286 showed a marked increase in the fermentation efficiency at 20 % initial sugar, but no significant increase in the utilization of substrate was observed. At 25 % (w/v) initial sugar concentration, both the substrate utilization and fermentation efficiency decreased in all the strains.

Fermentation pattern of Z. mobilis on various substrates

Synthetic medium: Figure 1 and table 3 show the fermentation pattern of 4 strains of *Z. mobilis* at optimum conditions in synthetic medium. Eventhough NRRL B 4286 and IFO 13756 showed low fermentation efficiency till 12 h, the fermentation efficiency was higher compared to others (ATCC 10988 and ATCC 12526). But IFO 13756 rapidly

Table 1. Effect of pH on ethanol production by various strains of *Z. mobilis*.

pH	Fermentation parameters	Strains			
		ATCC 10988	ATCC 12526	NRRL B 4286	IFO 13756
4.0	P	2·7	2·2	4·0	3·5
	Su	74·4	68·1	85·6	75·0
	g/gs	0·18	0·16	0·23	0·23
	E	26·37	21·48	39·06	34·18
5.0	P	2·9	3·2	4·2	4·4
	Su	ND	ND	87·5	81·3
	g/gs	ND	ND	0·24	0·27
	E	28·32	31·25	41·02	42·97
6.0	P	3·9	4·0	4·3	4·7
	Su	80·6	81·2	87·5	88·1
	g/gs	0·24	0·25	0·25	0·27
	E	38·09	39·06	41·99	45·9
7.0	P	6·6	6·3	5·2	6·4
	Su	82·77	79·0	88·1	92·5
	g/gs	0·40	0·40	0·30	0·35
	E	64·45	64·51	50·29	62·5

P, Productivity of alcohol % (v/v); Su, substrate utilized % (w/v); g/gs, gram alcohol produced/g of substrate utilized; E, percentage of theoretical yield; ND, not determined.

Table 2. Effect of initial sugar concentration on ethanol production by various strains of *Z. mobilis*.

Initial sugar (% w/v)	Fermentation parameters	Strains			
		ATCC 10988	ATCC 12526	NRRL B 4286	IFO 13756
15	P	6·6	6·3	5·1	6·1
	Su	82·77	79·0	88·0	93·3
	g/gs	0·43	0·44	0·31	0·33
	E	68·75	65·63	53·13	63·54
20	P	5·9	6·1	10·15	8·85
	Su	73·0	75·0	89·0	91·0
	g/gs	0·32	0·33	0·46	0·39
	E	46·09	47·65	79·29	69·14
25	P	5·2	5·5	7·9	7·2
	Su	76·0	78·0	84·8	75·6
	g/gs	0·22	0·23	0·30	0·30
	E	32·05	34·38	49·38	45·0
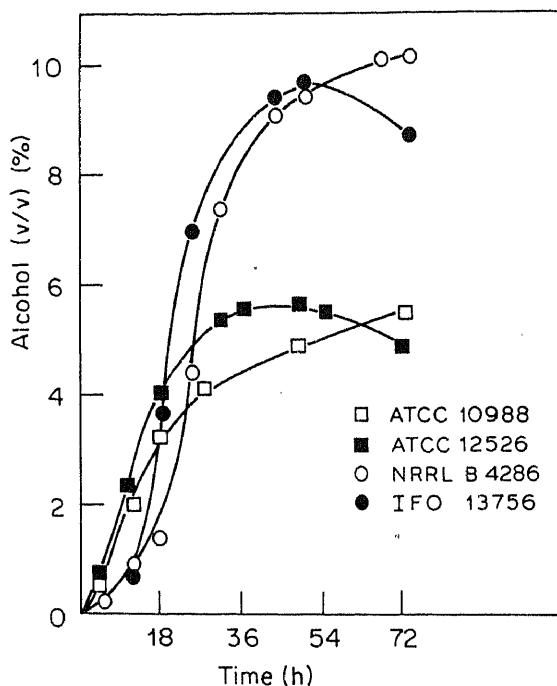
Figure 1. Ethanol production by strains of *Z. mobilis* on synthetic medium.

Table 3. Ethanol production by strains of *Z. mobilis* on various substrates.

Substrate	Fermentation parameters	Strains			
		ATCC 10988	ATCC 12526	NRRL B 4286	IFO 13756
Synthetic medium	P	5·9	6·1	10·15	8·85
	Initial sugar	20·0	20·0	20·0	20·0
	Su	73·0	75·0	89·0	91·0
	g/gs	0·32	0·33	0·46	0·39
	E	46·09	47·65	79·29	69·14
Cane juice	P	10·3	10·3	9·2	10·3
	Initial sugar	18·0	18·0	18·0	18·0
	Su	95·0	95·0	97·2	97·2
	g/gs	0·48	0·48	0·42	0·47
	E	89·4	89·4	79·86	89·4
Molasses	P	3·05	2·9	3·29	1·97
	Initial sugar	10·0	10·0	10·0	10·0
	Su	ND	ND	ND	ND
	g/gs	ND	ND	ND	ND
	E	47·66	45·31	51·41	30·78

fermented after 12 h and the fermentation ceased at 48 h and thereafter the ethanol content decreased. This could be due to the oxidation of alcohol to acetic acid (Belaich and Senez, 1965; Swings and DeLey, 1977). Among all 4 strains of *Z. mobilis*, NRRL B 4286 showed higher fermentation efficiency (10·15%, v/v) in synthetic medium. The specific productivity of ethanol was maximum (0·46) in this strain and minimum in ATCC 10988 (0·32).

Cane juice: Figure 2 and table 3 show the fermentation pattern of 4 strains of *Z. mobilis* in cane juice. The strain which showed better fermentation in synthetic medium (NRRL B 4286) was a poor fermentor in cane juice compared to others. This strain showed a delay of 12 h while in the others no such delay was observed. The strain ATCC 10988 rapidly fermented and fermentation ceased at 48 h. Lyness and Doelle (1981) reported that ethanol yield of *Z. mobilis* Z 7 in cane juice medium was 60–88 % in 20–29 h. The present study shows that the fermentation of ATCC 10988 lasted for 48 h and resulted in 82–94% ethanol. The fermentation continued upto 66 h in strain ATCC 12526 resulting in 10·3 % (v/v) ethanol.
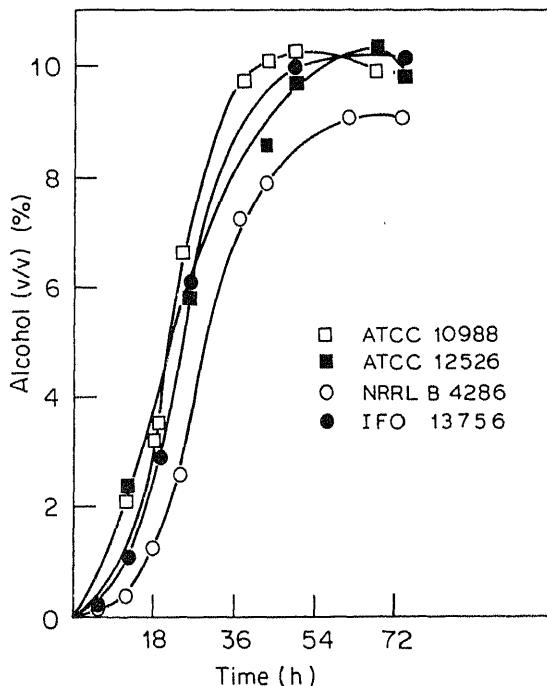
Figure 2. Ethanol production by strains of *Z. mobilis* on cane juice.

Molasses: Table 3 shows the ethanol production by strains of *Z. mobilis* in molasses with 10% (w/v) initial sugar concentration. The strains ATCC 10988 (3·05% v/v) and NRRL B 4286 (3·29% v/v) showed maximum productivity whereas IFO 13756 showed minimum productivity (1·97% v/v). *Z. mobilis* strain ATCC 10988 was well studied for its performance at different initial sugar concentrations of molasses (table 4). It showed

Table 4. Ethanol production at different initial sugar concentration of molasses by *Z. mobilis* strain ATCC 10988.

Initial sugar (% w/v)	Ethanol production (% v/v)	Theoretical yield (%)
3	1·25	65·1
5	2·0	62·5
7	2·5	55·8
10	3·05	47·66
15	3·35	34·9

maximum ethanol production (3·35%, v/v) at 15% (w/v) initial sugar concentration where substrate utilization was also found to be maximum (42% w/v). Above 15% (w/v) initial sugar concentration of molasses, the ethanol production decreased (data not shown). In general, in all 4 strains of *Z. mobilis*, the ability to produce ethanol from molasses was very poor compared to other substrates. This observation is consistent with the result of Van Vuuran *et al.* (1982) and this could be due to the presence of high concentration of ions (Mg^{2+} and K^+) in molasses (Skotnicki *et al.*, 1982). Furthermore, investigations are in progress to improve the fermentation ability of *Z. mobilis* in molasses.

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Carbon starvation mediated changes in carbohydrate metabolism in *Neurospora crassa*

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Abstract. Carbon starvation conditions were found to increase the activities of gluconeogenic enzymes such as malic enzyme, cytosolic malate dehydrogenase and isocitrate lyase along with proteases and inhibition in glucose catabolic enzymes such as G6P dehydrogenase and FDP aldolase in *Neurospora crassa*.

Keywords. *Neurospora crassa*; carbohydrate metabolism; starvation.

Introduction

A number of studies on starvation are concerned with situations in which traces of nutrients are available transiently (Novitsky and Morita, 1977; Jones and Rhodes-Roberts, 1981). Responses to such conditions of starvation include the development of specific systems for chemotaxis, to obtain scarcely available nutrients (Geesey and Morita, 1979), miniaturization of cells (Casida, 1977) and upkeep of readily available machinery for macromolecular synthesis (Koch, 1971). Starvation mediated changes have also been implicated as a decrease in the rate of protein synthesis (Calzone *et al.*, 1983) and accumulation of reserve materials (Matin *et al.*, 1979). The present study deals with the effect of starvation on changes in carbohydrate metabolism in *Neurospora crassa*.

Materials and methods

The carotenogenic strain of *N. crassa* (wild type) was obtained from the Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi, and was maintained on Saboraud's agar slants. The composition of the synthetic medium employed was the same as that described earlier (Acharya and Chhatpar, 1981). The culture was grown in 50 ml liquid medium in 250 ml Erlenmeyer flasks on a rotary shaker (180 rpm) at 30°C for 96 h. The mycelia were harvested by filtration and stored at − 5°C before use. For starvation experiments, *N. crassa* was grown for 72 h, the mats were removed aseptically, washed with sterile distilled water and transferred to

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either complete medium (normal condition) or to a medium lacking carbon sources (carbon starvation condition) and incubated for 24 h. Resupplementation was done by transferring the mats again to normal medium under aseptic conditions. The incubation was continued for 24 h. Filter-sterilized cycloheximide was added at 0·5 μg/ml.

For enzyme assays, a cell-free extract was prepared in 0·05 M Tris-HCl buffer, pH 7·2 by grinding the frozen mycelia mixed with glass powder in a chilled mortar. The extract was centrifuged at 15,000 *g* for 30 min at 4°C. The supernatant solution was used for cytosolic enzyme assays.

The assay methods used for FDP aldolase (fructose-1,6-diphosphate D-glyceraldehyde 3-phosphate-lyase, EC 4.1.2.13), malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) glucose-6-P dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49), isocitrate lyase (threo-D-isocitrate glyoxylate lyase, EC 4.1.3.1) and malic enzyme (L-malate: NADP oxidoreductase (decarboxylating) EC 1.1.1.40) were the same as described by Jagannathan *et al.* (1956), Ochoa (1955a) Kornberg and Horecker (1955), Dixon and Kornberg (1959) and Ochoa (1955b), respectively. Protease activity was assayed by the method of Ong and Gaucher (1973). The substrate used for neutral protease was 0·5 % casein in 0·1 M Tris-HCl buffer (pH 7·2) and for alkaline protease, 0·5 % casein in 0·1 M Tris-HCl buffer (pH 8·6). Protein and keto acids were determined according to the methods of Lowry *et al.* (1951) and Friedemann (1957), respectively. Lipids were extracted by the method of Folch *et al.* (1957) and estimated according to the method of Bragdon (1951). Polyacrylamide gel electrophoresis was carried out according to the procedure of Davis (1964). Activity staining of glucose-6-P dehydrogenase and malate dehydrogenase was carried out by the method of Corman *et al.* (1967).

Results and discussion

N. crassa when grown in a normal medium for 72 h in shake culture and transferred to starvation conditions for 24 h, showed a loss of FDP aldolase and G6P dehydrogenase activities (table 1). Activity staining on polyacrylamide gels for G6P dehydrogenase showed 3 prominent bands under normal conditions while 3 very faint bands with identical electrophoretic mobilities were detectable under conditions of carbon

Table 1. Effect of carbon starvation on the activities of FDP aldolase, G6P dehydrogenase, malic enzyme and the levels of keto acids and lipids in *N. crassa*.

Conditions of growth	FDP aldolase U/mg protein	G6P dehydrogenase U/mg protein	malic enzyme U/mg protein	Keto acids mg/mg protein	Lipids g/100 g dry wt.
Normal	119	136	255	0·06	3·01
Carbon starvation	ND	ND	962	0·02	1·7

ND, Not detectable; U, units. Units for FDP aldolase and G6P dehydrogenase are described as the amount of enzyme which brings about a change of 0·001 absorbance at 240 and 340 nm/min respectively at 30°C.

starvation (figure 1). In order to examine whether there was any possibility of an increase in the levels of these enzymes, if starved cultures are resupplemented with carbon source, starved cultures were transferred to normal medium. Under these conditions, a significant increase in the activity of FDP aldolase was observed suggesting the possibility of *de novo* synthesis of this enzyme in response to starvation-refed conditions (table 2). Starvation has been found to reduce the activities of a number of enzymes in rat liver (Szepesi *et al.*, 1975). Szepesi and Berdanier (1971) have shown a decrease in the activities of G6P dehydrogenase and malic enzyme by starvation in rats. When starved animals were refed with a diet rich in carbohydrates, activities of liver G6P dehydrogenase and malic enzyme rose significantly.
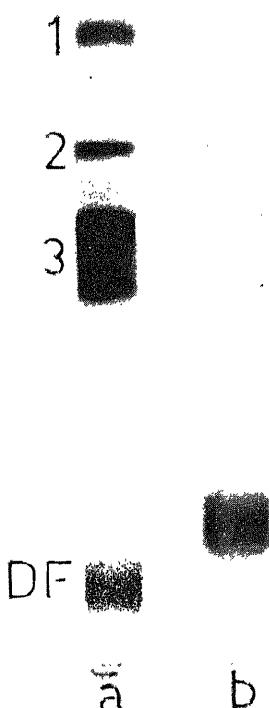
Figure 1. Activity staining for G6P dehydrogenase after polyacrylamide gel electrophoresis. (a), Normal condition; (b), carbon starvation condition. DF indicates Dye Front.

Table 2. Effect of carbon starvation and refeeding on the activities of isocitrate lyase, malate dehydrogenase and FDP aldolase in *N. crassa*.

Conditions of growth	Isocitrate lyase U/mg protein	Malate dehydrogenase U/mg protein	FDP aldolase U/mg protein
Normal	104	20	119
Carbon starvation	407	283	ND
Refeeding of carbon starved cells	98	164	262

ND, Not detectable. Units for isocitrate lyase and malate dehydrogenase are described as the amount of enzyme which brings about a change of 0·001 absorbance at 324 and 340 nm respectively per min at 30°C.

Since proteases are known to play a significant role in turnover of proteins, attempts were made to determine the levels of proteases in starvation. There was a significant increase in the activity of both neutral and alkaline proteases (table 3). In order to examine whether there was *de novo* synthesis of proteases, starvation was carried out in the presence of cycloheximide. There was no increase in the activity of neutral protease or alkaline protease under these conditions suggesting that proteases might have been synthesized in response to carbon starvation conditions. Drucker (1972) has also shown the production of protease in response to carbon starvation conditions. Hanson and Marzluf (1975) have shown that when *Neurospora* is grown in starvation medium in which an endogenous protein serves as the major or sole carbon source, it synthesizes and secretes a protease into the medium.

Table 3. Effect of carbon starvation in the presence and absence of cycloheximide on the activities of G6P dehydrogenase and neutral and alkaline proteases in *N. crassa*.

Conditions of growth	G6P dehydrogenase (U/mg protein)	Neutral protease (U/mg protein)	Alkaline protease (U/mg protein)
Normal	55	0·1	0·2
Carbon starvation	ND	0·3	0·5
Carbon starvation in the presence of cycloheximide	25	ND	0·2

ND, Not detectable. Unit of proteases is defined as μmol of tryptophan liberated per hour at 37°C.

In *N. crassa*, other enzymes of carbohydrate metabolism such as isocitrate lyase, cytosolic malate dehydrogenase (table 2) and malic enzyme (table 1) were studied in normal and carbon starved conditions. All these enzymes showed an increase in activity during starvation. Activity staining of polyacrylamide gels for malate dehydrogenase showed two prominent bands under carbon starvation conditions as compared to two very faint bands observed under normal conditions (figure 2). These are gluconeogenic enzymes. These results suggest that under starvation conditions, gluconeogenesis is favoured. To substantiate these results, refeeding experiments were carried out to measure isocitrate lyase, malate dehydrogenase and FDP aldolase levels. Isocitrate lyase and malate dehydrogenase showed a considerable decline in activity when starved cultures were resupplemented with normal medium. FDP aldolase on the contrary
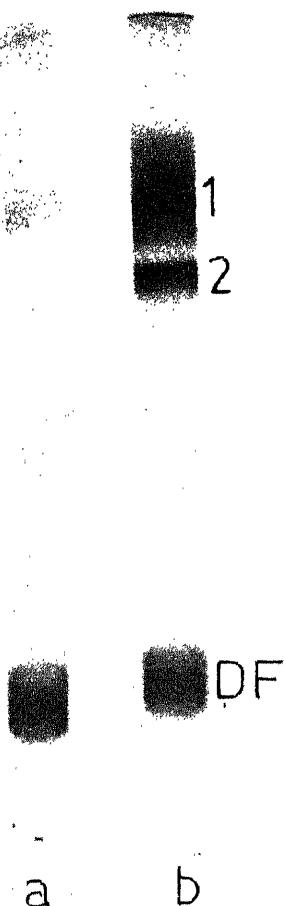
Figure 2. Activity staining for malate dehydrogenase after polyacrylamide gel electrophoresis. (a), Normal condition; (b), carbon starvation conditions. DF indicates Dye Front.

showed an increase in the activity suggesting the need for this enzyme under these circumstances.

Lipids and keto acids were also found to show substantial changes under carbon starvation conditions (table 1). The decrease in the levels of G6P dehydrogenase, an enzyme important for the generation of reducing power may suppress lipogenesis. The lipid content was found to be 42% less under carbon starvation conditions.

Carbon starvation requires energy conservation and for replenishment small metabolic products are rechanneled in an organised way for better survival conditions. The decrease in catabolic enzymes such as FDP aldolase and G6P dehydrogenase and an increase in anabolic enzymes such as malate dehydrogenase, malic enzyme and isocitrate lyase fulfill these requirements.

Acknowledgement

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Correlation between riboflavin carrier protein induction and its mRNA activity in estrogen stimulated chicken liver and oviduct

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Abstract. Poly A enriched RNA from either liver or oviduct of estradiol-17β treated immature chicks supported [^{3}H]-leucine incorporation into immunoprecipitable riboflavin carrier protein in a dose-dependent manner when translated in the rabbit reticulocyte lysate system. Primary translation product of riboflavin carrier protein had a molecular weight of 38,000 which on incubation with a stripped hepatic microsomal preparation was processed to a product with a size comparable to native riboflavin carrier protein. Poly A enriched RNA from both the liver and the oviduct of estrogen-treated birds stimulated [^{3}H]-leucine incorporation into riboflavin carrier protein and this was 2–3 fold higher during secondary stimulation *vis-a-vis* primary stimulation with the steroid. Poly A enriched RNA from the liver of progesterone-treated birds during secondary stimulation did not support riboflavin carrier protein synthesis. In contrast, poly A enriched RNA from the oviduct of the birds treated with progesterone during secondary (but not primary) stimulation did exhibit riboflavin carrier protein-mRNA activity which was comparable to that stimulated by estradiol-17β.

Keywords. Primary translation product; cell-free translation; rabbit reticulocyte lysate; stripped microsomal membrane; progesterone; estradiol-17β; precursor; poly A$^+$-RNA.

Introduction

According to current concepts of mechanisms of steroid hormone-induced specific gene expression in eukaryotic cells, one of the major events consequent on functional interaction of the hormone-receptor complex with the genomic regulatory sites on the chromatin is the initiation and accelerated transcription of protein-specific mRNA (Chan *et al.*, 1978). In line with the above is the finding that there exists excellent correlation between elevated levels of specific mRNAs and enhanced production of proteins encoded by them. This is amply illustrated by the estrogenized avian (Deeley *et al.*, 1975) and amphibian (Tata, 1976) liver expressing *de novo* the vitellogenin gene and the steroid-induced production of several egg white proteins by the differentiated chicken oviduct (Palmiter, 1975). Our earlier investigations on estrogen-induced *de novo* expression of the gene encoding riboflavin carrier protein (RCP) in the chicken

liver (Murthy and Adiga, 1978) and oviduct (Durga Kumari and Adiga, 1986) have clearly shown that, despite gross similarities between the inductive responses in the two tissues, there are subtle differences with regard to the kinetics and modulation of induction of this vitamin-carrier in the two hormone-responsive organs. However, these conclusions were arrived at largely by measurement of plasma and tissue levels of RCP by radioimmunoassay (RIA) which reflect, the total contents of this protein (which in turn may be influenced by the turnover rate) and hence indirectly the biosynthetic potency of the respective tissue. It was therefore considered desirable to assay the levels of RCP-mRNA in the two tissues to correlate the same with their RCP contents. Towards this end, the translational efficiency of isolated poly A enriched RNA (poly A$^+$-RNA) preparations from the avian liver and oviduct in a heterologous cell-free system was exploited as a direct index of mRNA contents (Wetekam *et al.*, 1975). Incidently, this also provided an opportunity to examine the nature of the primary translational product of RCP gene in the two avian tissues. The data presented in this paper reveal that the primary translational product of the RCP gene in the two estrogen-responsive avian tissues are grossly similar. Further, they indicate that there exists a significant correlation between the tissue mRNA levels and their capacity to elaborate RCP in response to hormonal stimuli.

Materials and methods

ATP, GTP, creatine phosphate, creatine phosphokinase, spermidine HCl, ethylene glycol-bis (2-amino ethyl ether) N,N'-tetraacetic acid (EGTA), dithiothrietol (DTT), N-2 hydroxy ethyl piperazine-N'-2 ethane sulphonic acid (HEPES) and *Micrococcal* nuclease were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Oligo (dT)-cellulose was purchased from Collaborative Research Inc., Waltham, Massachusetts, USA. [^{3}H]-Leucine (specific activity, 136 Ci/m mol) was purchased from the Radio-chemical Centre, Amersham, Bucks, UK. All other chemicals and reagents were of analytical grade. The source of the White Leghorn chicks and their maintenance have been described earlier (Murthy and Adiga, 1978; Muniyappa and Adiga, 1980). The chicken RCP was purified to homogeneity from the egg white and specific anti bodies were raised in rabbits as described earlier (Murthy and Adiga, 1977).

Hormone regimen

A group of female chicks (2-week-old, 150 g body wt) were injected (intramuscular) daily with estradiol-17β (10 mg/kg body wt/day) in propylene glycol for 10 days during primary stimulation. After 21 days of hormone withdrawl, they were subdivided into two groups and were administered either estradiol-17β or progesterone, (both at 10 mg/kg body wt/day) respectively for 4 days (secondary stimulation).

Isolation of chicken liver and oviduct poly A$^+$-RNA

Extraction of polysomal RNA and preparation of poly A$^+$-RNA: Polysomes were prepared by the procedure of Palmiter (1974). Birds were sacrificed 24 h after the last hormone injection following both primary and secondary stimulations. Livers and

oviducts quickly excised from decapitated birds were homogenized in 9 volumes of 0·025 M Tris-HCl (pH 7·5) buffer containing 0·025 M NaCl, 5 mM $MgCl_2$, 2 % Triton X-100 and 1·0 mg/ml of heparin. After centrifugation at 27,000 g for 5 min at 4°C, the supernatant was mixed with an equal volume of the homogenising buffer containing 0·2 M $MgCl_2$. After standing for 2 h in the cold (4°C), the supernatant was layered over one half volume of 0·025 M Tris-HCl (pH 7·5) containing 0·025 M NaCl, 5 mM $MgCl_2$ and 0·5 M sucrose and centrifuged at 27,000 g for 20 min. The supernatant was aspirated and the polysomal pellet was used for the extraction of RNA.

RNA was prepared from the polysomes by extraction with phenol-chloroform (Palmiter, 1974).

Poly A^+-RNA was isolated from the polysomal RNA by chromatography on oligo dT-cellulose (Krystosek *et al.*, 1975). Briefly, a column (2·5 ml bed volume) of regenerated oligo (dT)-cellulose was used and equilibrated with 20 ml of high salt-sodium dodecyl sulphate (SDS) buffer [0·01 M, Tris-HCl (pH 7·5), 0·5 % SDS, 0·5 M NaCl]. RNA dissolved in high salt-SDS buffer (Ca 15–20 A_{260} units/ml) was applied to the column which was washed successively with the high salt-SDS buffer and then with 0·01 M Tris-HCl (pH 7·5) 0·5 M NaCl. The poly A^+-RNA was eluted with autoclaved distilled water. The RNA in the effluent was precipitated with 2·5 volume of 95 % ethanol as before. It was dissolved in a minimal volume of autoclaved distilled water and stored at −20°C until used.

Protein synthesis in rabbit reticulocyte cell-free system

The reticulocytes were isolated (Palmiter, 1973) from rabbits rendered anaemic by treatment with phenylhydrazine hydrochloride. The washed cells were finally lysed with one volume of autoclaved distilled water and centrifuged at 10,000 g for 20 min at 4°C. Haemin (25 μM) and creatine phospho kinase (50 μg/ml) were added to the lysate and stored at −70°C.

Endogenous globin mRNA in the reticulocyte lysate was removed by digesting briefly (9 min at 20°C) with *Micrococcal* nuclease (Pelham and Jackson, 1976) in the presence of $CaCl_2$ (1 mM). The nuclease was inactivated with EGTA (2 mM) and the lysate directly used for the translation of chicken liver and oviduct poly A^+-RNAs.

Preparation of stripped microsomal membranes

These were prepared by the method of Blobel and Dobberstein (1975) with slight modifications. Liver dissected from control (unstimulated) chicks were rinsed with and homogenized in the buffer containing triethanolamine (50 mM, pH 7·5), 0·25 M sucrose, 50 mM KCl, 5 mM $MgCl_2$ and 2 mM DTT. The homogenate was clarified by centrifugation at 10,000 g for 10 min and the supernatant was spun at 105,000 g at 4°C for 1 h. The microsomal pellet thus obtained was suspended in ice-cold 50 mM Tris-ethanolamine-HCl (pH 7·4)/50 mM KCl to a concentration of 100 A_{280} units/ml. EDTA was added to a final concentration of 1·5 μ mol/10·0 A_{280} units and the suspension layered on 25 % sucrose in 50 mM triethanolamine-HCl (pH 7·4)/50 mM KCl buffer. After centrifugation for 2 h at 105,000 g, the pellet comprising of EDTA-stripped microsomes was resuspended in 50 mM triethanolamine-HCl (pH 7·4)/50 mM KCl and centrifuged again at 105,000 g for 1 h. The pellet was

suspended in 20 mM HEPES buffer, (pH 7·6) and was treated with *Micrococcal* nuclease as described earlier to remove the traces of RNA sticking to the membranes.

Translation of poly A$^+$-RNA

The incubation mixture (100 μl) contained 40 μl of rabbit reticulocyte lysate, 0·02 M HEPES buffer (pH 7·6), 1 mM DTT, 1 mM ATP, 100 μm GTP, 10 mM creatine phosphate, 375 μM spermidine-HCl, 16 μCi of [^{3}H]-leucine, 50 mM potassium acetate, 1 mM magnesium acetate, 50 μM phenyl methyl sulphonyl fluoride and chicken liver or oviduct poly A$^+$-RNA. The mixture was incubated at 37°C for 60 min. In some cases, the stripped microsomal membranes were included at a concentration of 5·0 A$_{280}$ units/ml. After 60 min, the tubes were centrifuged at 15,000 g for 5 min to remove any insoluble material. Aliquots were taken for the measurement of labelled amino acid incorporated into trichloroacetic acid-insoluble protein (Mans and Novelli, 1961) by liquid scintillation spectrometry.

Immunoprecipitation of radioactive RCP

After incubation, labelled lysate samples were adjusted to a final concentration of 0·2 % SDS, 10^{-4} M EDTA, 1% sodium deoxy cholate (w/v), 1% Triton X-100 (v/v) and 0·1 M Tris-HCl (pH 7·5) in a total volume of 0·4 ml and the purified chicken RCP (10 μg) was added as the carrier. To this, 0·1 ml RCP-antiserum was added, to provide a 2-fold excess of the antibody on the basis of the equivalence point. After incubation for 1 h at room temperature and for 12 h at 4°C, the immunoprecipitate was collected by centrifugation at 15,000 g for 5 min. It was washed 4 times with the buffer containing 1% sodium deoxycholate, 1% Triton X-100, 10^{-4} M EDTA and 0·1 M Tris-HCl (pH 7·5). The immunoprecipitate was suspended in buffer containing 10 mM Tris-HCl (pH 7·5), 2 × 10^{-4} M EDTA and 150 mM NaCl. This was layered over 1 M sucrose cushion and spun at 15,000 g for 5 min. The pellet was washed twice with the same buffer. Immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 7% gels (Laemmli, 1970).

Radioactivity measurements

After PAGE and staining with Coomassie brilliant blue R-250 the gels were sliced (2 mm) and digested with 0·5 ml of 30 (w/v) H$_2$O$_2$ at 55°C for 15 h. The immuno-precipitates to be counted directly were dissolved in 0·1 ml of 90% formic acid, transferred to Whatman No. 3 filter paper discs, dried and counted using toluene based scintillator containing 0·5 % (w/v) 2,5 diphenyl oxazole (PPO). For aqueous samples (gel digests) a mixture of 2-methoxy ethanol and toluene (1:1 v/v) containing 0·5% PPO was used as the scintillation fluid. Samples were counted in a Rack beta liquid scintillation counter (LKB Produktor, Sweden).

Results

Significant incorporation of [^{3}H]-leucine into immunoprecipitable RCP could be clearly observed in the rabbit reticulocyte cell-free translation system when poly A$^+$-

RNA from either the liver or the oviduct of hormone-treated, (but not control) chicks was used as the exogenous source of mRNA. When poly A^+-RNA from either of the avian tissues was added, protein synthesis was linear upto 60 min (data not given). Under optimal conditions of translation, *i.e.*, 59 mM K^+, 1 mM Mg^{2+} and incubation time of 60 min, poly A^+-RNA (8 μg/100 μl) from hormone treated chicken liver stimulated [^{3}H]-leucine incorporation into total proteins 8–10 fold while that from the oviduct 10–14 fold.

Labelled RCP immunoprecipitated using the specific antibody was analysed on SDS-PAGE to ascertain the size distribution of the primary translation product. It could be shown that RCP synthesized in the heterologous cell-free system had a molecular weight (M_r) of 38,000; (pre-RCP); there was no detectable labelling in the region of mature RCP (M_r 36,000) (figure 1). It is significant that the total mRNA from the control chicks showed no detectable radioactivity at positions corresponding to either of these protein bands. The total poly A^+-RNA fraction from estrogen-treated
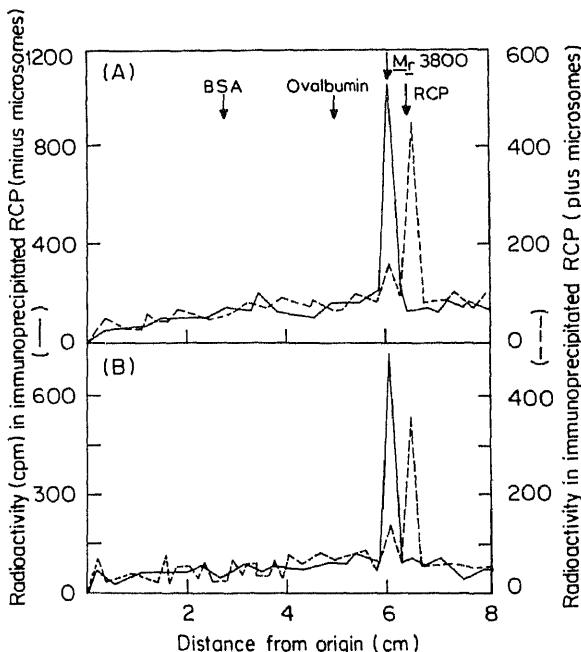
Figure 1. Characterization of [^{3}H]-RCP synthesized in the reticulocyte lysate system programmed with poly A^+-RNA from estradiol-17β treated chicken oviduct and liver. Optimal concentrations of poly A^+-RNAs isolated from chicken oviduct (A) and liver (B) were translated in the heterologous cell-free system and in the presence and absence of stripped microsomes. [^{3}H]-RCP synthesized was immunoprecipitated using 2-fold excess of specific RCP antiserum. Washed immunoprecipitates were subjected to SDS-PAGE and gels stained and scanned for radioactivity. Bovine serum albumin, ovalbumin and RCP served as internal markers. For other details see 'materials and methods'. The positions of radioactivity peaks on the gels were fixed relative to RCP which obviated inter gel variations in the mobilities of protein species.

chicken oviduct during primary stimulation also showed a pattern similar to that elicited by the liver mRNA (figure 1). When the translation was carried out in the presence of 'stripped' microsomal membranes, from control chicken liver, there was a significant decrease in the radioactivity associated with M_r 38,000 pre-RCP species, with a concomitant increase in radioactivity in the region that moved marginally ahead of the native RCP. This slightly faster mobility of the microsomal membrane-processed translation product relative to native RCP may be attributed to the lack of covalently attached carbohydrate moiety. This is presumably due to the inability of the lysate system to glycosylate the primary translation product. A similar observation was made earlier in the case of ovalbumin mRNA (Rhodes *et al.*, 1971) translated in the reticulocyte lysate system.

The translatable RCP-mRNA activities from estrogenized chicken liver and oviduct during primary stimulation and from the corresponding tissues from estrogen-primed but secondarily stimulated with either estrogen or progesterone for 4 days were assayed to assess the magnitude of stimulation of RCP gene expression. The results of figure 2 indicate that at equal RNA concentrations $(8\,\mu g/100\,\mu l)$, the poly A^+-RNA from
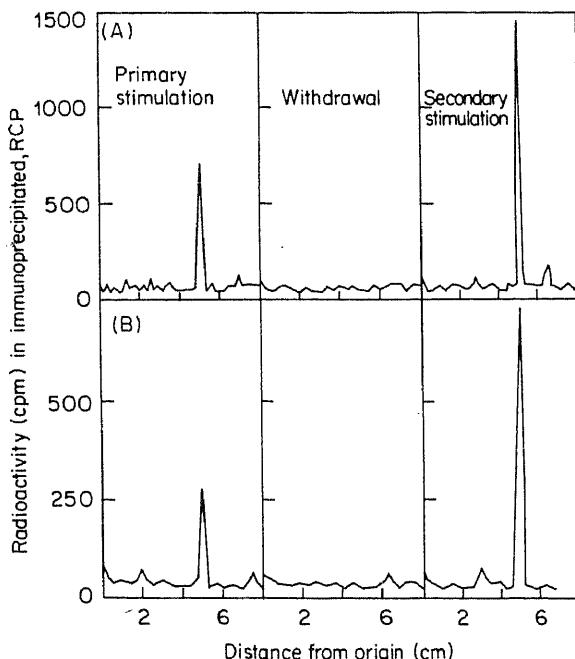
Figure 2. Distribution of radioactivity in electrophorograms following resolution of immunoprecipitated [^{3}H]-RCP on SDS-PAGE. [^{3}H]-RCP synthesized in the rabbit reticulocyte lysate system programmed with equivalent amounts of poly A^+-RNA from estrogen-treated chicken oviduct (A) and liver (B) during primary stimulation, hormone withdrawal and secondary stimulation were immunoprecipitated and subjected to SDS-PAGE. Gels were scanned for radioactivity and protein stain. Radioactivity in the region corresponding to M_r 38,000 was quantitated. For other details see legend to figure 1.

oviducts of chicks treated with estrogen during secondary stimulation promoted pre-RCP synthesis almost 2-fold more than that from the oviducts of chicks during primary stimulation (table 1). Similarly liver poly A^+-RNA isolated from birds, secondarily stimulated with estrogen enhanced RCP synthesis by 2·7 fold more than that obtained during primary stimulation (figure 2). Interestingly, there was no detectable synthesis of pre-RCP, when poly A^+-RNA from livers or oviducts of either control birds or birds withdrawn from the hormone were used (figures 1 and 2).

The significance of the finding was that poly A^+-RNA from liver treated with progesterone during secondary stimulation failed to support pre-RCP synthesis to any discernible extent whereas poly A^+-RNA from the oviducts of estrogen-primed chicks stimulated secondarily with progesterone did support pre-RCP synthesis more or less to the same extent as that from oviducts of estrogen-primed chicks treated again with estrogen during secondary stimulation (table 1).

Table 1. Effect of estradiol-17β and progesterone of immature female chicks on translatable RCP mRNA activity in the oviduct and liver during primary and secondary stimulations.

Source of poly A^+-RNA		Protein synthesis (cpm)		RCP (% of total protein) synthesized
Group	Tissue	Total ($\times 10^{-4}$)	RCP	
E_I	Liver	20·00	2400	1·2
	Oviduct	24·10	2000	0·83
$E_I \rightarrow E_{II}$	Liver	23.20	7130	3·1
	Oviduct	28·00	5880	2·1
$E_I \rightarrow P_{II}$	Liver	16·20	20	0·0
	Oviduct	24·10	4800	2·0

Three groups of 5 female chicks (15 day old) were administered with estradiol-17β (10 mg/kg body wt/day) for 10 consecutive days (primary stimulation). One group was sacrificed and their livers and oviducts were processed for poly A^+-RNA. The remaining two groups were withdrawn from estrogen for 21 days and each of these groups were administered (secondary stimulation) either estradiol-17β or progesterone (each at 10 mg/kg body wt/day) for 4 consecutive days. Poly A^+-RNA prepared from their oviducts and livers were translated in the rabbit reticulocyte lysate system and [³H]-RCP immunoprecipitated and quantified as described under 'materials and methods'. E_I, E_{II} represent primary and secondary stimulations with estradiol-17β while P_{II} represents secondary stimulation with progesterone.

These results are in good agreement with the corresponding data obtained by measurements of liver of oviductal cytosolic RCP levels quantitated either by RIA or labelled amino acid incorporation into tissue explants (Durga Kumari and Adiga, 1986). This provides confirmatory evidence that progesterone could stimulate RCP mRNA activity only in the differentiated oviduct but not in the liver.

Discussion

The first indication, that RCP mRNA does in fact accumulate intracellularly in the steroid hormone-stimulated avian tissues, stems from the earlier observation that both the relative and absolute rates of RCP syntheses increase with time after hormonal

dosing (Durga Kumari and Adiga, 1986). Motivated by a desire to obtain a direct correlation between the extent of RCP induction and intracellular levels of mRNA activity, the versatile heterologous cell-free translation system *viz.*, the rabbit reticulocyte lysate, was recruited for mRNA quantification. The availability of a potent and highly specific RCP-antibody was exploited to measure RCP synthesized *in vitro* by immunoprecipitation. It could be clearly established that the lysate system responds to poly A^+-mRNA preparation from either chicken liver or oviduct in dose-dependent manner. Poly A^+-RNA preparations from the unstimulated (control) birds fail to support RCP synthesis in the cell-free system which confirms our earlier finding that RCP gene is not constitutively expressed, but induced *de novo* in response to the hormonal stimuli (Murthy and Adiga, 1978).

It is significant that assayable RCP-mRNA activity becomes clearly manifest in both the tissues during primary stimulation with estrogen, but not with progesterone and this activity declines to undetectable levels upon hormone withdrawal (figure 2). It is also clear (table 1) that equivalent amounts of poly A^+-RNA from the oviducts of estrogen-primed birds given either estrogen or progesterone during secondary stimulation, support RCP synthesis 2-fold higher than mRNAs extracted from the tissues during primary stimulation. This clearly confirms the results obtained with the measurement of the oviduct content of RCP by RIA (Durga Kumari and Adiga, 1986) which not only showed the equipotency of the two steroids in terms of stimulating RCP synthesis/unit wt. of the tissue, but also correlated the magnitude of the amplified response elicited during secondary stimulation with estrogen (Murthy and Adiga, 1978). It is noteworthy that progesterone administration either during primary or secondary stimulation did not elicit any detectable RCP mRNA activity in the liver which is in conformity with the total lack of inductive response as reflected by undetectable plasma levels of RCP. It is also pertinent to mention at this juncture that earlier investigations with the chicken oviduct from hormone-withdrawn birds did reveal the presence, though in vastly reduced amounts of mRNAs specific to both conalbumin and ovalbumin (McKnight and Palmiter, 1979) (as measured by cDNA hybridization technique) but were inactive, since they were not associated with the polysomes. But the present study employing a cell-free translation system failed to reveal detectable amount of RCP-mRNA activity in both the liver and the oviduct under conditions of hormone withdrawal (figures 1 and 2). Most probably, this may be related to the fact that RCP represents a much smaller proportion (1–2 %) of the total protein synthesized both in the oviduct and the liver (Durga Kumari and Adiga, 1986) than conalbumin (10 %) or ovalbumin (50–60 %). Alternatively, mRNA translation assay is not as sensitive as the cDNA-hybridization technique earlier employed to quantitate ovalbumin and conalbumin mRNAs (McKnight and Palmiter, 1979).

It may however be emphasized that the accumulation of RCP-mRNA, encountered during the different hormonal stimulations, may reflect both the enhanced transcription of RCP gene and significant cytoplasmic stabilization as has been observed earlier during vitellogenin induction in the avian (Gruber *et al.*, 1976) and amphibian liver (Brock and Shapiro, 1983) as well as during egg white protein production by the chicken oviduct (McKnight and Palmiter, 1979). The relative contributions of these two phenomena to the overall accumulation of mRNA activity are unknown at present and await further study.

The approach of mRNA quantification using the heterologous cell-free translation system (with no apparent post-translational modification) also afforded an opportunity to compare the nature of the primary translation product of RCP mRNAs from the two avian tissues. The finding that there is no gross numerical or size differences in the immunoprecipitable-labelled protein recovered, supports the earlier premise (Farrell *et al.*, 1966) that a single structural gene encodes this specific protein in two different cell types. Similar conclusions have been arrived at regarding translational and transcriptional products of transferrin gene in the two avian tissues (McKnight *et al.*, 1980). It would appear therefore, that tissue specific differential hormonal regulation of RCP gene expression may not be attributable to the recruitment of different copies of the same gene family as has been demonstrated in the case of genes coding for MUP proteins (Shaw *et al.*, 1983) and α_{2u} globulin (Laperche *et al.*, 1983) in murine liver and submaxillary glands.

The information regarding the primary translational product of RCP has some relevance in the context of the proposed secondary structure of RCP. It was hypothesized earlier (Clagett, 1971) that like insulin, RCP consists of two unequal subunits held covalently by two disulphide bonds and hence its biosynthesis might involve a precursor (pro-RCP?) which in turn may be processed proteolytically to native RCP on lines demonstrated for proinsulin → insulin conversion (Steiner and Oyer, 1967). The above translation data (figure 1) no doubt reveal that RCP synthesized in the rabbit reticulocyte lysate has a larger (M_r 38,000) size, which clearly distinguishes it from the native RCP. While this accords with the postulates of Clagett (1971) regarding involvement of a precursor in RCP synthesis, it is rather doubtful whether further processing of this slightly larger translation product proceeds on the lines suggested for insulin biosynthesis. Infact, the data recorded here seem to favour the view that the primary translation product of RCP represents pre-RCP rather than pre-pro-RCP (based on the nomenclature adopted for intermediates in insulin biosynthesis), since on incubation with stripped microsomal membrane preparation, it could be readily converted to an entity with a size comparable to native RCP (figure 1) without the carbohydrate rather than to a molecule with an expected size intermediate between the two, as has been observed during insulin processing. In other words, the processing of the primary translation product on incubation with the 'stripped' microsomal membranes follows Blobel hypothesis and is more akin to the cleaving of the 'signal peptide' from the new synthesized secretory proteins (Blobel and Dobberstein, 1975). It may also be emphasized that our repeated attempts to separate the two putative subunits of RCP by SDS-PAGE under reducing conditions have borne negative results (Murthy *et al.*, 1976). Thus bulk of our current evidence seem to suggest that this specific protein is biosynthesized as 'pre-RCP' but not as 'pre-pro-RCP' unlike as envisaged earlier (Clagett, 1971). In line with these observation is the recent finding that both chicken egg yolk and white RCP are single chain polypeptides with identical amino sequence and hence are products of a single structural gene (Narioka *et al.*, 1985).

Acknowledgements

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Decarboxylation of arginine and ornithine by arginine decarboxylase purified from cucumber (*Cucumis sativus*) seedlings

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Abstract. A purified preparation of arginine decarboxylase from *Cucumis sativus* seedlings displayed ornithine decarboxylase activity as well. The two decarboxylase activities associated with the single protein responded differentially to agmatine, putrescine and P_i. While agmatine was inhibitory (50 %) to arginine decarboxylase activity, ornithine decarboxylase activity was stimulated by about 3-fold by the guanido amine. Agmatine-stimulation of ornithine decarboxylase activity was only observed at higher concentrations of the amine. Inorganic phosphate enhanced arginine decarboxylase activity (2-fold) but ornithine decarboxylase activity was largely uninfluenced. Although both arginine and ornithine decarboxylase activities were inhibited by putrescine, ornithine decarboxylase activity was profoundly curtailed even at 1 mM concentration of the diamine. The enzyme-activated irreversible inhibitor for mammalian ornithine decarboxylase, *viz.* α-difluoromethyl ornithine, dramatically enhanced arginine decarboxylase activity (3–4 fold), whereas ornithine decarboxylase activity was partially (50 %) inhibited by this inhibitor. At substrate level concentrations, the decarboxylation of arginine was not influenced by ornithine and *vice-versa*. Preliminary evidence for the existence of a specific inhibitor of ornithine decarboxylase activity in the crude extracts of the plant is presented. The above results suggest that these two amino acids could be decarboxylated at two different catalytic sites on a single protein.

Keywords. Arginine decarboxylase; purification; ornithine decarboxylase; modulation; DFMO; dual activities.

Introduction

In the past, the involvement of ornithine decarboxylase (ODC) (EC 4.1.1.17) in putrescine biosynthesis in the plant systems was generally considered to be of minor consequence. However, in recent years, pronounced ODC activities have been demonstrated in a number of plant systems such as *Helianthus tuberosus* (Bagni and Speranza, 1977), tomato ovary cells in culture (Cohen *et al.*, 1982), oat leaf protoplasts (Flores and Galston, 1982), peas (Dai *et al.*, 1982), potatoes (Kaur-Sawhney *et al.*, 1982), *Phaseolus vulgaris* (Palavan and Galston, 1982), mung bean seedlings (Altman *et al.*, 1982) and barley (Kyariakidis, 1983) during experimental manipulation. Furthermore, two pathways for putrescine biosynthesis, involving both arginine decarboxylase (ADC) (EC 4.1.1.19) and ODC have been detected in *Agrobacterium tumefaciens* and in

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Abbreviations used: ODC, Ornithine decarboxylase; ADC, arginine decarboxylase; DFMA, difluoromethyl arginine; DFMO, difluoromethyl ornithine; PLP, pyridoxal phosphate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; NEM, N-ethylmalieimide.

normal and crown gall tissues of *Scorzonera hispanica* (Speranza and Bagni, 1977). Recently, Kyriakidis *et al.* (1983) have purified ODC from barley seedlings and found that a large portion of enzyme activity was localized in the nuclear fraction and the remainder in the cytoplasm. With the exception of this study, detailed investigations on the properties of plant ODC are meagre. The possibility that ODC rather than ADC, could be an important regulatory enzyme in plants under certain conditions was suggested by the finding that in tomato ovary cells during fruit setting and XD-cells of tobacco (Cohen *et al.*, 1982; Heimer *et al.*, 1979), ODC activity was enhanced nearly 30-fold while ADC activity remained unchanged. Similarly, during profuse sprouting of potato tubers (Kaur-Sawhney *et al.*, 1982), a 6-fold increase in ODC activity was encountered suggesting that ODC may be relatively of greater importance in this plant system also.

Recently, Prasad and Adiga (1985b) purified ADC from cucumber (*Cucumis sativus*) seedlings and studied some of its molecular properties. Although ODC activity could not be assayed in crude extracts of this plant system, it was intriguing to find that homogenous ADC preparation obtained from the plant seedlings, efficiently catalysed enzymic decarboxylation of ornithine also. In this paper, we provide evidence to support this observation and describe the responses of the dual enzyme activities associated with the purified protein to various ligands, besides comparing some of the properties of these two catalytic functions associated with the single protein.

Materials and methods

Difluoromethyl arginine (DFMA) and difluoromethyl ornithine (DFMO) were generous gifts of Merrell Dow Chemical Company, Cincinnatti, Ohio, USA. Sources of cucumber seeds, the method of their germination and seedling growth and of other chemicals were the same as Prasad and Adiga (1985b). [1-^{14}C]-Ornithine (Sp. activity 59 mCi/m mol) was obtained from Radiochemical Centre, Amersham, England, while [U-^{14}C]-L-arginine (Sp. activity 246 mCi/m mol) was obtained from Bhabha Atomic Research Centre, Bombay.

Purification of ADC

ADC from cucumber seedlings was purified according to a 3-step procedure developed in this laboratory (Prasad and Adiga, 1985b) involving ion exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-150. The protein fraction eluted in the void volume possessed ADC and ODC activities and was homogenous upon electrophoresis at pH 8·3 or 4·3 on 7·5% polyacrylamide gels respectively (figure 1).

ADC could also be purified by a combination of ion exchange chromatography and affinity chromatography on organomercurial Sepharose (Prasad and Adiga, 1985b). The physicochemical properties and electrophoretic behaviour of both the preparations are the same.

Enzyme assays

Assays of ADC/ODC were carried out in Warburg flasks as detailed elsewhere (Ramakrishna and Adiga, 1975). Briefly, the reaction mixture for measurement of ADC
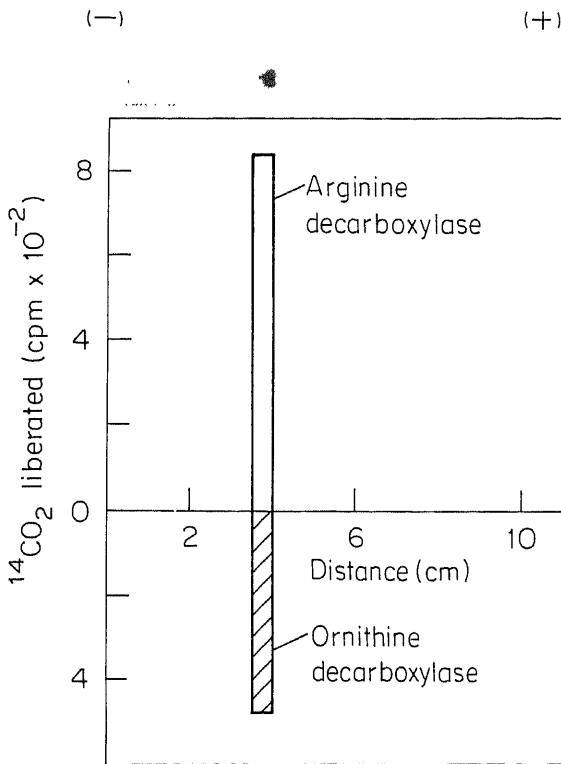
Figure 1. PAGE of ADC and ODC of *C. sativus.*
 After electrophoresis (at pH 8·3) of the purified enzyme at 4°C, the distribution of ADC and ODC activities was monitored by sectioning the gels and eluting at 4°C in 20 mM Tris-HCl pH 7·6 containing 2 mM 2-mercaptoethanol and 10 μM PLP. The enzyme activities were measured in the supernatant after removing the gel pieces by centrifugation.

activity consisted of 0·1 M Tris-HCl, pH 8·2, 2·5 mM dithiothreitol, 50 μM pyridoxal phosphate (PLP) and 200,000 cpm of L-[U-^{14}C] arginine (0·36 μM). The reaction was initiated by the addition of substrate and incubated at 40°C for 2 h. Quantification of $^{14}CO_2$ liberated during the post incubation period was done as described earlier (Ramakrishna and Adiga, 1975).

Assay of ODC was also conducted as described above using same reaction components but the labelled arginine was replaced by [1-^{14}C]-ornithine (200,000 cpm, 1·5 μM). Ommission of unlabelled amino acid in the assay mixture did not alter our enzyme assay values. Similar observations have been made earlier by Goren *et al.* (1982). Blank values (without the addition of the enzyme) were routinely subtracted from the enzyme assay values.

Unit activity

One unit of enzyme activity is defined as that which liberates 1 pmol of $^{14}CO_2$/2 h at 40°C. Specific activity is expressed in units/mg of protein.

Other methods

Polyacrylamide gel electrophoresis (PAGE) at pH 8·3 was carried out according to Davis (1964). PAGE at pH 4·3 on 5 % gels was carried out as described by Reisfield *et al.* (1962). Sodium dodecyl sulphate (SDS) PAGE was performed according to Laemmli (1970). Antibodies were raised aginst the purified protein and double immunodiffusion on agar was performed as described by Ouchterlony (1967). Protein estimation was carried out according to Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

Results

Criteria of purity of ADC

PAGE: The Sephadex G-150 eluate moved as a single stainable band during electrophoresis under non-denaturing conditions at pH 8·3 (figure 1) and 4·3 (data not given). When subjected to SDS-PAGE, under non-reducing conditions, the protein again moved as a single band (data not given).

Immunological criterion: When the antibodies raised against the Sephadex G-150 eluate of the protein were allowed to interact with the purified protein, a single precipitin line was observed (data not shown).

Co-elution of ADC and ODC acitivities during gel filtration: When the Sephadex G-150 eluate (containing ADC activity) was tested for its substrate specificity, it was intriguing to find that ornithine also could be decarboxylated enzymatically by the same preparation efficiently. Since these two decarboxylating activities associated with the purified protein were excluded from Sephadex G-150, the possibility was considered that the dual enzyme activities might be due to a minor contaminant in the enzyme protein preparation. Therefore an attempt was made to separate them by further gel filtration on Sephacryl S-300. Under this condition also, both the activities were found to elute in the exclusion volume and the protein thus obtained could decarboxylate either arginine or ornithine in proportion to protein concentrations (table 1). The product of ornithine decarboxylase, putrescine could be identified on chromatograms (data not given).

Table 1. Decarboxylation of arginine and ornithine in Sephacryl S-300 eluted protein (purified ADC from cucumber seedlings).

Enzyme source	Activity units	
	ADC	ODC
Sephadex G-150 eluate		
65 μg protein	4·2	7·8
Sephacryl S-300 eluate		
55 μg protein	3·6	7·7
110 μg protein	7·7	15·8

The purified enzyme upon electrophoresis at pH 8·3 resolved into a single stainable protein band. When a duplicate gel was sliced (5 mm pieces) and individual slices were assayed for both the enzyme activities, both ADC and ODC activities banded at a region coincident with protein stain (figure 1). Both ADC and ODC assays were linear upto 200 μg protein included in the assay mixture and 2 h of incubation time. ADC activity followed Michaelis-Menten kinetics with a $K_{m\,ARG}$ 0·5 mM with V_{max} of 5·5 n mol/mg protein/h. ODC also followed a similar pattern with K_m for ornithine being 0·028 mM and V_{max} of 277 p mol/mg protein/h. Both the decarboxylating activities had a pH optima of 8·2 (data not shown).

Modulation of ADC and ODC activities by phosphate and agmatine

When agmatine, the immediate product of ARG-decarboxylation was included in the assay mixture, ADC activity was inhibited by 41 % (table 2) whereas ODC activity was significantly stimulated (2·5 fold) under this condition. Addition of inorganic phosphate stimulated ADC activity by 2-fold, while ODC activity was only marginally affected. When the effect of agmatine on ODC activity was tested at various concentrations, a linear dose-response could not be obtained (figure 2). Agmatine at lower concentrations *i.e.*, upto 2·5 mM had no discernible effect while at 5 mM concentration was stimulatory to ODC activity. It is noteworthy that the selective stimulatory influence of agmatine on ODC and a similar effect of P_i on ADC could be observed only if the freshly purified enzyme protein was used; aged enzyme preparations, while retaining significantly both the activities, were unaffected by these modulators for reasons not easily understood at present.

Table 2. Modulation of arginine and ornithine decarboxylation by P_i and agmatine in Sephadex G-150 eluate (pure ADC preparation from cucumber seedlings).

Additions (mM)	Activity of control (%)	
	ADC	ODC
Nil (control)	100[a]	100[a]
Agmatine (10)	59	241
P_i (5·0)	182	116

[a] Control values for ADC and ODC are 82 and 170 activity units/mg protein respectively.

P_i and agmatine were included in the assay mixture at indicated concentrations.

Effect of putrescine

Putrescine inhibited both ODC and ADC activities but to different extents (figure 2). The diamine at lower concentrations (1 mM) did not influence ADC activity; even at 10 or 15 mM concentrations, a maximum of about 53 % of enzyme activity was obtained. Further inhibition was not observed on increasing putrescine concentration (figure 2).
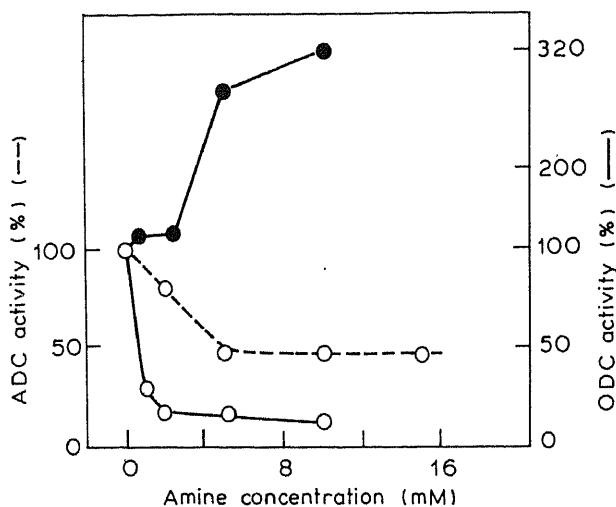
Figure 2. Effect of agmatine and putrescine on ADC and ODC activities of *C. sativus.*
Agmatine (●) and putrescine (O) were included to the ADC (‒‒‒‒) or ODC (‒‒‒‒) assay
mixture at concentrations indicated and the enzyme activities are estimated as described in
'materials and methods'.

In marked contrast, ODC activity was clearly more susceptible to putrescine inhibition,
since as low as 1 mM of the diamine caused 70 % inhibition and almost complete
inhibition was achieved with 10 mM of putrescine.

Effect of DFMO

This enzyme-activated irreversible inhibitor of mammalian ODC (Metcalf *et al.*, 1978)
exerted differential influence on ADC and ODC activities associated with the purified
protein. When preincubated, the enzyme with DFMO at as low as 1 mM markedly
enhanced ADC activity (data not given). Thus, preincubation for 5 min resulted in
3-fold increase in ADC activity which was further enhanced to 4-fold when this period
was extended to 10 min. Its concurrent effect on ODC activity was however clearly
marginal (data not shown). Thus preincubation for 5 min at 1 mM DFMO was
ineffective in inhibiting ODC activity to any discernible extent, whereas at 2 mM and
on preincubation for 20 min about 50 % inhibition of ODC activity could be elicited.
Moreover, it could be clearly shown that this type of influence of DFMO on both ADC
and ODC activities persisted even after dialysis. When the enzyme was preincubated
with DFMO and assayed for ADC in the presence of 10 mM unlabelled ornithine,
ADC activity was still elevated. When ODC activity was assayed in the presence of
10 mM arginine, after preincubation with DFMO, enzyme was inhibited to greater
extent (table 3).

Effect of citrulline and homoarginine

ADC activity was inhibited approximately to 60 % by 10 mM citrulline, while ornithine
decarboxylation was almost completely suppressed under these conditions.

Table 3. Effect of DFMO on ADC/ODC activity from cucumber seedlings.

Assay conditions	Activity units/mg protein	
	ADC	ODC
Control	28	79
DFMO[a]	78 (278)	34 (43)
DFMO[a] + dialyzed[b]	78 (278)	33·5 (42)
DFMO[a] + ornithine 10 mM	82 (292)	N.D.[c]
DFMO[a] + arginine 10 mM	N.D.[c]	11·5 (14)
Control + arginine 10 mM	N.D.[c]	0[d]
Control + ornithine 10 mM	0[d]	N.D.[c]

Values in parentheses are per cent activity of control.
[a] Enzyme was preincubated with 2 mM DFMO for 20 min in a buffer containing 10 mM Tris-HCl, pH 7·6, 2 mM mercaptoethanol and 10 μM PLP. The final concentration of the inhibitors in the assay was 0·1 mM.
[b] Preincubated ezyme was dialyzed (against 500 volumes for 3 h at 4°C) and assayed for activity.
[c] Not detectable due to dilution.
[d] Enzyme activity inhibited.

Homoarginine was also effective in curtailing both the ODC and ADC activities (table 4). Lysine when tested at 5 mM concentration did not serve as a substrate for the enzyme under the assay conditions employed (data not shown).

Table 4. Effect of homoarginine and citrulline on ADC and ODC activities from cucumber seedlings.

Additions (mM)	Activity units/mg protein	
	ADC	ODC
Nil (control)	100	100
Citrulline (10)	41 (59)[a]	0 (100)[a]
Homoarginine (10)	4 (96)[a]	0 (100)[a]

[a] Values represent per cent inhibition.
Citrulline and homoarginine were added to ADC/ODC standard assay mixture. Assay was started by adding Sephadex G-150 eluate (pure ADC preparation).

Effect of pyridoxamine, $NaBH_4$, semicarbazide, NEM and KCl

Addition of either $NaBH_4$ (50 mM) or NEM (10 mM) completely inhibited both ADC and ODC activities, indicating essential requirements for carbonyl function and sulphydryl groups for catalysis. Pyridoxamine (20 mM) inhibited ADC activity to 64 % and that of ODC to 43 % while as low as 20 mM KCl was effective in inhibiting both the activities to comparable extents (table 5).

Table 5. Effect of KCl, pyridoxamine, sodium borohydride and NEM on ADC and ODC activities from cucumber seedlings.

Additions (mM)	Activity units/mg protein	
	ADC	ODC
Nil (control)	72	171
KCl (20)	59 (19·9)[a]	120 (30)[a]
Pyridoxamine (20)	26 (64)[a]	98 (43)[a]
NaBH$_4$ (50)	0 (100)[a]	10 (94·2)[a]
NEM (10)	0 (100)[a]	15 (92)[a]

[a] Values represent per cent inhibition.
   The additions were included in the standard assay mixture for measuring ADC/ODC activity.

Effect of arginine and ornithine on the decarboxylation of labelled ornithine and arginine respectively

When both labelled arginine (0·36 n mol) and ornithine (1·54 nmol) were included simultaneously in the assay mixture, it was found that both of them were efficiently utilized as substrates, since the amount of $^{14}CO_2$ liberated was more or less comparable to that arising out of individual enzyme activities (data not given). In other words, the presence of one of the unlabelled amino acid at the concentrations indicated did not seem to greatly affect the utilization of the other radioactive amino acid as the substrate.

Cryptic nature of ODC in crude extracts

Data of table 6 clearly show that the ODC activity in crude extracts or during early purification steps was undetectable. The enzyme could be assayed only in the purified preparations. Upon mixing the crude extracts with the purified enzyme, ODC activity was found to be significantly inhibited.

Table 6. ODC activity of cucumber seedlings during various stages of purification.

Enzyme source (μg of protein)	ODC activity units/mg of protein
Crude extracts (460)	Not detectable
MnCl$_2$ supernatant (200)	Not detectable
DEAE-Cellulose eluate (200)	Not detectable
Sephadex G-150 eluate (120)	136
Sephadex G-150 eluate (120) + crude extract (230)	17
Sephadex G-150 eluate (120) + crude extract (460)	6·0

   Crude extracts and the purified enzyme (Sephadex G-150 eluate) were mixed before starting the enzyme assay.

Discussion

The most significant observation in the present study, is the clearcut demonstration for the first time that the purified ADC from the cucumber seedlings has intrinsic ODC activity associated with it. It may be recalled that in prokaryotes where ADC and ODC activities co-exist and function in putrescine production under appropriate physiological conditions, their activities are not only associated with entirely different proteins, but are modulated differentially under various conditions (Morris and Fillingame, 1974; Pegg and Williams-Ashman, 1981). In the past, there have been a few reports describing specific amino acid decarboxylases which utilize other related amino acids as substrates. For example, in *Lathyrus sativus* seedlings, an enzyme catalyzes the decarboxylation of both homoarginine and lysine, though with different efficiencies (Ramakrishna and Adiga, 1976). In rat tissues, Pegg and McGill (1979) have shown that ODC could decarboxylate lysine when ODC activity was induced to a high level and lysine concentrations exceeded substantially those of ornithine. Similar results were obtained by Persson (1981) using testosterone-treated mouse kidney as the enzyme source. Furthermore, it was demonstrated that the two decarboxylating activities copurified with a constant ratio of specific activities and responded to different inhibitors in an analogous fashion in the mouse kidney extracts. Repeated attempts during the present study to quantify ODC activity either in crude extracts or during the earlier stages of purification were unsuccessful and the mechanisms underlying this cryptic nature of the ODC activity are currently ill-understood. Since, in mammalian and microbial systems, ODC activity can be masked by macromolecular inhibitors (Canellakis *et al.*, 1979), it is conceivable that similar inhibitory factors (which are apparently lost upon purification) may contribute to our inability to assay ODC activity in cucumber crude extracts. The fact that ODC activity was not discernible in DEAE-cellulose eluate but became assayble in the final preparations favours such a view. Mixing experiments also lend credence to the above premise (table 6). The low specific activity of ADC preparation is due to the instability of the enzyme preparations, proteolytic degradation and tendency of the enzyme to get inactivated as the purification progressed (Prasad and Adiga, 1985b). Since ODC activity is not detectable until the final stage of purification no further information can be given; moreover, it was not possible to determine the specific activity ratios of ADC and ODC.

That the dual activities associated with the purified enzyme may not be mediated through involvement of a single active site, is suggested by differential modulatory influence exerted by the various effectors of the two enzyme activities tested. Interestingly, the extent of inhibition (50 %) of purified ADC by agmatine under the assay conditions employed in this compares favourably with that encountered *in vivo* in agmatine treated cucumber cotyledons maintained in organ culture (Prasad and Adiga, 1985a). Phosphate-activation of ADC with no concurrent comparable influence on ODC as well as agmatine-stimulation of ODC activity attended by simultaneous profound inhibition of ADC are in line with the above premise. However, it may be pointed out that these regulatory characteristics are apparently labile since they were lost on aging of the enzyme. This is in accordance with the observation that regulatory/allosteric properties of several enzymes are rendered labile to such processes as proteolytic modification, dilution etc. (Reddy *et al.*, 1980). It is also conceivable that

significant activation of ODC achieved with agmatine, above a threshold value (5 mM) could be a physiologically relevant control mechanism, in the sense that when agmatine accumulates beyond a certain critical intracellular level, ODC pathway of putrescine production may be favoured with concomitant inhibition of ADC, thereby sparing arginine for other competing vital cellular processes such as protein synthesis. This hypothesis implicates agmatine as a 'check post' for arginine flow into polyamine production in the plants.

Another line of evidence which is suggestive of involvement of different active site conformations for the catalytic efficiency of ADC and ODC activities associated with the same protein, stems from the differential extents of inhibition of activities by putrescine (figure 2). Near complete inhibition of ODC activity by the diamine in this plant system contrasts with the situation encountered with the mammalian enzyme where the diamine is only a weak competitive inhibitor (McCann *et al.*, 1977). On the other hand, less efficient curtailing effect of putrescine on ADC activity compares favourably with the result obtained with ADC purified from *L. sativus* (Ramakrishna and Adiga, 1975).

Another rather intriguing aspect of this bifunctional enzyme is related to modulation of the two constituent activities by their respective irreversible inhibitor *viz.*, DFMO. The observation that the maximum inhibition of this plant ODC activity by DFMO was confined to around 50% contrasts with the situation encountered with the mammalian enzyme (Mamont *et al.*, 1980) but has a qualitative parallelism in some microbial systems. It may be recalled that ODC from both *Escherichia coli* and *Klebsiella pneumoniae* (Kallio and McCann, 1981) are refractory to this suicide enzyme inhibitor. That the latter aspect of similarity between the microbial and the cucumber enzyme extends to concurrent profound activation of ADC activity is clearly evident from the data of table 3. It is significant that DFMO-facilitated arginine-decarboxylation is sustained even in the presence of excess ornithine which is otherwise highly inhibitory to ADC activity (table 3). Intriguingly, in the presence of excess arginine, otherwise limited (50%) inhibition of ODC activity by DFMO is significantly enhanced (90%).

Despite these subtle differences in the modulatory aspects of the two decarboxylase activities associated with the protein, other results clearly support the notion that both the activities belong to the general category of amino acid decarboxylases. Thus, both the activities are sensitive to inhibition by NEM and $NaBH_4$ and to a large extent by pyridoxamine indicating thereby an obligatory requirement for sulphydryl function and carbonyl groups. Results obtained with EDTA and Mn^{2+} clearly eliminate the possibility of the arginase + urease type (Lignowski and Splittstoesser, 1971) of reactivity contaminating the purified enzyme.

It appears that both ADC and ODC activities localized on the same protein have their individual domains that are interdependent in certain respects. The advantages that could possibly accrue to the plant in such an organization of dual activities on the protein molecule are several, among which concentration-dependent regulation of the individual enzyme activities by the respective substrates and common modulators may be the most crucial in the selection of the suitable enzymic pathway of putrescine synthesis under appropriate conditions.

Acknowledgements

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The binding requirements of monkey brain lysosomal enzymes to their immobilised receptor protein

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Abstract. The lysosomal enzyme binding protein (receptor protein) isolated from monkey brain was immobilised on Sepharose 4B and used to study the binding of brain lysosomal enzymes. The immobilised protein could bind β-D-glucosaminidase, α-D-mannosidase, α-L-fucosidase and β-D-glucuronidase. The bound enzymes could be eluted either at an acid pH of 4·5 or by mannose 6-phosphate but not by a number of other sugars tested. Binding could be abolished by prior treatment of the lysosomal enzymes with sodium periodate. Alkaline phosphatase treatment of the enzymes did not prevent the binding of the lysosomal enzymes to the column but decreased their affinity, as seen by a shift in their elution profile, when a gradient elution with mannose 6-phosphate was employed. These results suggested that an 'uncovered' phosphate on the carbohydrate moiety of the enzymes was not essential for binding but can enhance the binding affinity.

Keywords. Lysosomal enzyme binding protein; brain; immobilisation; binding requirements.

Introduction

In fibroblasts, lysosomal acid hydrolases are targeted to lysosomes by a membrane bound receptor which recognises mannose 6-phosphate residues on the enzymes (Natowicz *et al.*, 1979; von Figura and Klein, 1979; Ullrich *et al.*, 1978; Distler *et al.*, 1979). The phosphomannosyl receptor has been purified from bovine liver (Sahagian *et al.*, 1981) and from rat chondrosarcoma (Steiner and Rome, 1982). Like the fibroblast receptor these purified receptors also recognise mannose 6-phosphate on the lysosomal enzymes. Synthesis of the phosphomannosyl recognition marker involves the sequential action of two enzymes with the intermediate formation of a phosphodiester (lysosomal enzyme . . . mannose-6-(P)-1-N acetyl glucosamine) (or 'blocked' phosphate) followed by its cleavage to a phosphomonoester (Reitman and Kornfeld, 1981; Hasilik *et al.*, 1981; Varki and Kornfeld, 1980; Waheed *et al.*, 1981). Although the mannose 6-phosphate recognition marker has been shown essential for the uptake of lysosomal enzymes by fibroblasts, alternate mechanisms of recognition have been suggested by the following studies:

(i) Studies (Owada and Neufeld, 1982; Waheed *et al.*, 1982) on organs obtained from patients with I-cell disease have shown that although the N-acetylglucosamine-1-

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Abbreviation used: Con A, Concanavalin A.

phosphate transferase enzyme responsible for the phosphorylation of mannose was absent in this disease, normal or near normal levels of lysosomal enzymes were found in the brain, liver, kidney and spleen.

(ii) Gabel *et al.* (1983) have shown that mutant murine cell lines which lack the mannose 6-phosphate receptor are still capable of sequestering high levels of acid hydrolases in the lysosomes, thereby suggesting the existence of an alternate pathway for the entry of enzymes into the lysosomes. These mutant cells are now demonstrated to have a second type of cation dependent phosphomannosyl receptor (Hoflack and Kornfeld, 1985).

(iii) Talkad and Sly (1983) and Cladaras *et al.* (1983) have shown that the bovine liver receptor could also bind 'blocked' phosphate residues on the lysosomal enzymes.

(iv) Multivalent interactions involving some component of the protein backbone have been suggested to contribute to the enzyme receptor interaction (Karsen *et al.*, 1980; Myerowitz and Neufeld, 1981; Rome and Miller, 1980).

(v) Macrophages and liver non-parenchymal cells have been shown to recognise mannose and N-acetylglucosamine residues on lysosomal enzymes (Diment and Dean, 1983; Ullrich *et al.*, 1979).

(vi) Freeze (1985) showed that oligosaccharides with two phosphodiesters and the peptide portion of slime mold lysosomal enzymes are important for their recognition by the fibroblast phosphomannosyl receptor.

Hill *et al.* (1985) showed the uptake of β-glucosidase from *D. discoideum* and β-galactosidase from bovine testes by rat cerebral cortex astrocytes through mannose 6-phosphate receptors. Very little, however, is known about the biosynthesis and packaging of brain lysosomal enzymes in brain cells. We have previously reported the isolation by phosphomannan-Sepharose chromatography of a binding protein (receptor protein) for lysosomal enzymes from monkey brain (Alvares and Balasubramanian, 1983). The binding of brain lysosomal enzymes by this receptor protein was markedly inhibited by mannose 6-phosphate followed by mannose and N-acetylglucosamine. In this report we describe the interaction of 4 brain lysosomal enzymes with the receptor protein immobilised on Sepharose and demonstrate that the presence of an 'uncovered' phosphate on the carbohydrate moiety of the enzyme is not obligatory for binding to the receptor protein although it may enhance the binding affinity.

Materials and methods

Phenolphthalein glucuronide, *p*-nitrophenyl glycosides, *Escherichia coli* alkaline phosphatase (type III) and the sugars were from Sigma Chemical Co., St. Louis, Missouri, USA, sodium metaperiodate from J. T. Baker and Sepharose 4B from Pharmacia. Concanavalin A (Con A) was prepared from *Canavalia gladiata* and coupled to CNBr-activated Sepharose as described earlier (Surolia *et al.*, 1973; Alam and Balasubramanian, 1978). Endoglucosaminidase H (Seikagaku Kogyo Co., Japan) was a kind gift from Dr. K. von Figura, Federal Republic of Germany. All other chemicals were of the highest purity grade available.

Enzyme assays

β-D-Glucuronidase was assayed using phenolphthalein glucuronide as substrate (Alvares and Balasubramanian, 1982). N-Acetyl β-D-glucosaminidase, α-D-mannosidase and α-L-fucosidase were assayed using their respective *p*-nitrophenyl glycosides as substrates as described earlier (Alvares and Balasubramanian, 1983). One unit of enzyme activity is defined as 1 μmol of substrate hydrolysed in 1 h at 37°C under the standard assay conditions. Purified human serum pseudocholinesterase was prepared and assayed using butyryl thiocholine iodide as substrate (George and Balasubramanian, 1981). Protein was estimated according to Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

Isolation of the brain lysosomal enzyme binding protein and its immobilisation

The lysosomal enzyme binding protein was isolated from monkey brain by affinity chromatography on a phosphomannan–Sepharose column (Alvares and Balasubramanian, 1983).

The method of immobilisation involved the activation by CNBr of Sepharose 4B according to the method of March *et al.* (1974) and the coupling of the receptor protein to the activated Sepharose through a 6 carbon arm (George and Balasubramanian, 1981). The activated Sepharose (2 ml) was suspended in an equal volume of 0·1 M borate buffer pH 9·5 and 0·5 g hexanediamine in 2 ml of the same buffer was added to the gel. The pH was adjusted to 9·5 with 5 M HCl and the gel stirred at 4°C for 24 h. The excess amine was removed by washing the gel with 20 volumes of 1 M NaCl followed by water. The aminated gel was suspended in 2 ml of 20 mM potassium phosphate buffer, pH 5·0 and coupled to 1·3 mg of receptor protein in 2 ml of the same buffer in the presence of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide for 24 h at 4°C. The excess protein was removed by washing the gel with 1 M NaCl. The amount of protein coupled was 0·74 mg.

Chromatography of lysosomal enzymes on the receptor protein-Sepharose column

Con A-Sepharose eluate, rich in lysosomal enzymes, in 20 mM Tris-HCl, pH 7·4 was passed through the receptor-Sepharose column (3 × 0·7 cm) equilibrated with the same buffer, at a flow rate of 5 ml/h. The column was washed with 10 bed volumes of the same buffer and then eluted with either 20 mM citrate-phosphate buffer, pH 4·5 or 20 mM sugars in 20 mM Tris-HCl, pH 7·4. Fractions of 2 ml were collected and assayed for enzyme activity.

In other experiments, the column was eluted with a linear gradient of 0 → 35 mM mannose 6-phosphate in 20 mM Tris-HCl, pH 7·4 (20 ml in each chamber) at a flow rate of 2 ml/h. This was followed by an elution with 20 mM citrate-phosphate buffer, pH 4·5. Fractions of 1 ml were collected and assayed for enzyme activities.

Periodate treatment

Sodium metaperiodate was added to a final concentration of 10 mM to the enzyme previously dialysed against 20 mM phosphate buffer, pH 6·0. Incubation was carried out in the dark at 4°C for 8 h at which time sodium metaperiodate was once again

added bringing the final concentration to 20 mM and the incubation continued for another 16 h. The reaction was quenched by the addition of 0·2 volumes of 1 M ethylene glycol followed by dialysis against 20 mM Tris-HCl buffer, pH 7·4. Periodate treatment resulted in 10–15 % of loss in glucosaminidase, glucuronidase and mannosidase activity and complete loss in fucosidase activity.

Amino acid modifications

Lysine and cysteine residues were modified by trinitrobenzene sulphonic acid according to Fields (1972), tyrosine by N-acetyl imidazole (Riordan *et al.*, 1965), arginine by phenylglyoxal (Chang and Huang, 1981) and tryptophan by 2-hydroxy-5-nitrobenzyl bromide (Barman and Koshland, 1967).

Alkaline phosphatase treatment

E. coli alkaline phosphatase digestion was done by incubating 1·25 mg of the Con A-Sepharose eluate with 7·5 units of phosphatase in 1 ml of 40 mM Tris-HCl, pH 7·4 for 1 h at 37°C. The contents were then transferred to a dialysis bag and the incubation continued at 22°C for 2 h while dialysing against 1 litre of 40 mM Tris-HCl, pH 7·4 (Talkad and Sly, 1983). Alkaline phosphatase treatment resulted in a loss of approximately 32 %, 20 %, 38 % and 10 % activity for the enzymes glucosaminidase, fucosidase, mannosidase and glucuronidase, respectively.

Purification of β-glucuronidase and endoglucosaminidase H treatment

β-Glucuronidase from monkey brain was purified upto the stage of antibody-Sepharose column chromatography (Alvares and Balasubramanian, 1982). To 1 ml (0·65 units) of the purified glucuronidase in 20 mM sodium acetate buffer, pH 5·5 was added 0·1 ml (0·01 unit) of endoglucosaminidase H and incubated at 37°C for 48 h (Lang *et al.*, 1984). As control, β-glucuronidase was incubated for the same period without endoglucosaminidase H. There was about 80 % loss in glucuronidase activity during the 48 h incubation.

Determination of bound phosphate in the Con A-Sepharose eluate before and after E. coli alkaline phosphatase treatment

The Con A-Sepharose eluate (0·045 mg protein) was dialysed against water and digested with 0·7 ml of 70 % perchloric acid on a sand bath at 160°C for 2 h followed by the measurement of inorganic phosphate according to Galliard *et al.* (1965).

Results

Binding of lysosomal enzymes to the immobilised receptor protein

Figures 1 and 2 show the binding of brain lysosomal enzymes to the receptor protein-Sepharose column. The column was capable of binding at least 4 different lysosomal enzymes tested, glucosaminidase, fucosidase, glucuronidase and mannosidase. The bound enzymes could be eluted either by mannose 6-phosphate or at an acid pH of 4·5
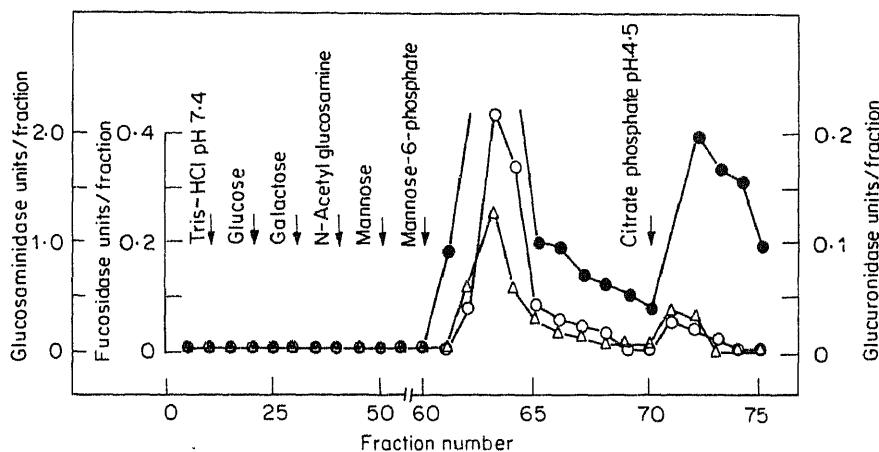
Figure 1. Binding of lysosomal enzymes to the receptor protein–Sepharose column and elution.

Con A-Sepharose eluate containing 68·4 units glucosaminidase (●), 6·8 units fucosidase (O) and 2·32 units glucuronidase (△) was loaded on the column and washed as described under 'materials and methods'. Mannosidase binding was not measured in this experiment. There was complete binding of all the enzymes. Arrows indicate the start of elution by 20 mM buffer or different sugars. Recovery of enzyme from the column was 16 % for glucuronidase, 19 % for fucosidase and 38 % for glucosaminidase. Recovery in this experiment was much lower than those following possibly because of the long time taken for elution by various sugars.

(figure 1). The rate of elution with mannose 6-phosphate appeared to be slow and incomplete when compared to the acid pH of 4·5. Glucose, galactose, mannose and N-acetylglucosamine at 20 mM could not elute the bound enzymes (figure 1).

Figure 2 shows the effect of periodate treatment (which results in the cleavage of carbohydrate residues) on the binding capacity of the enzymes. While all the enzymes bound to the column before periodate treatment (figure 2A), after periodate treatment 76 %, 100 % and 84 % respectively of glucosaminidase, mannosidase and glucuronidase did not bind and appeared in the breakthrough and wash fractions (figure 2B). Fucosidase lost all the activity on periodate treatment and so could not be monitored for binding.

The requirement of the carbohydrate portion of the lysosomal enzymes for their binding to the immobilised receptor protein was also confirmed using a single lysosomal enzyme namely β-glucuronidase of monkey brain. The purified β-glucuronidase (0·65 units) could completely bind to the column, but after periodate treatment all the enzyme activity remained unbound to the column. An alternate method of carbohydrate removal was done by limited digestion of the purified glucuronidase using endoglucosaminidase H as given under 'materials and methods'. Endoglucosaminidase H is known to cleave the carbohydrate portion from N-asparaginyl linked high mannose glycoproteins. Only limited digestion upto 48 h was possible because even by this period there was 80 % loss in glucuronidase activity. About 10 % of the digested glucuronidase remained unbound to the receptor protein–Sepharose column. We verified that only 10 % of glucuronidase had been
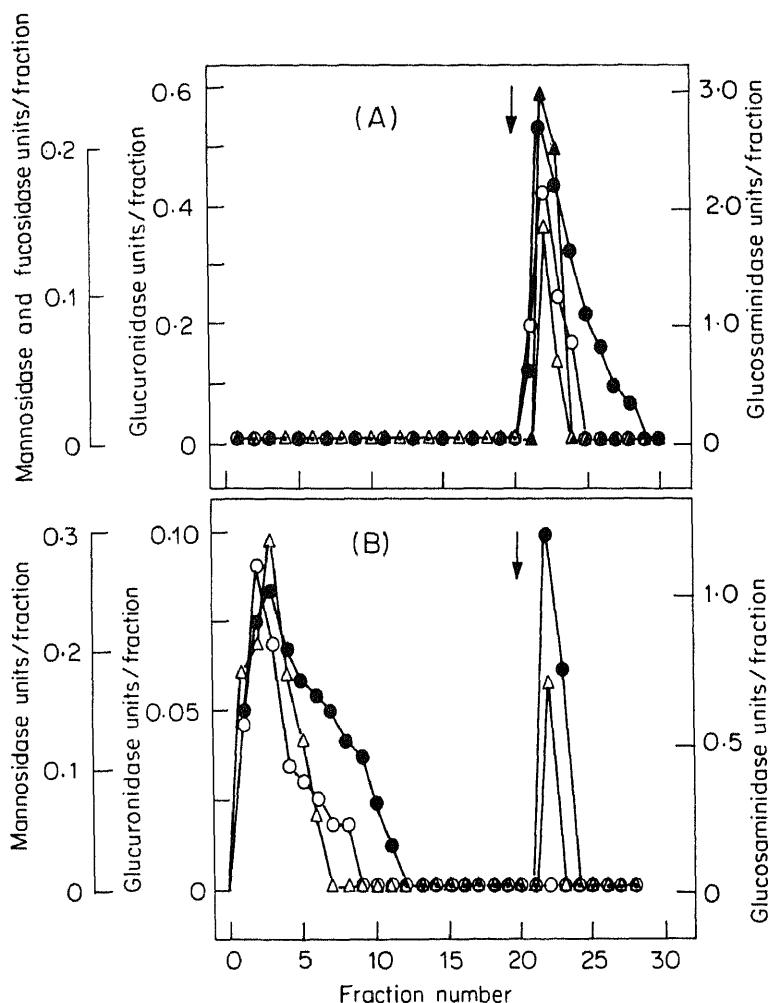
Figure 2. Effect of periodate treatment of lysosomal enzymes on binding to the receptor–Sepharose column.

A. Con A–Sepharose eluate (not treated with periodate) containing 12 units glucosaminidase (●), 0·72 units mannosidase (O), 0·72 units glucuronidase (Δ) and 0·75 units fucosidase (▲) was loaded on the receptor–Sepharose column, washed with 20 mM Tris–HCl pH 7·4 and eluted with citrate–phosphate buffer, pH 4·5. Recoveries of the enzymes were in range of 58–76 %.
B. Periodate treated Con A–Sepharose eluate containing 12·4 units glucosaminidase (●), 1·32 units mannosidase (O) and 0·66 units glucuronidase (Δ) was loaded on the receptor–Sepharose column, washed with 20 mM Tris–HCl pH 7·4 and eluted with citrate–phosphate buffer, pH 4·5. Recoveries of the enzymes were in the range of 62–72 %. Fucosidase lost all the activity after periodate treatment and so could not be measured. Arrow indicates start of elution.

deglycosylated by endoglucosaminidase H treatment by passing the digested glucuronidase through a Con A–Sepharose column (which binds mannose residues of glycoprotein) whereby the same percentage of enzyme was found to remain unbound to

the column. Untreated glucuronidase completely bound to the same Con A–Sepharose column, while periodate treated glucuronidase remained completely unbound.

Alkaline phosphatase treatment and gradient elution with mannose 6-phosphate

Treatment with *E. coli* alkaline phosphatase has been used by several investigators to dephosphorylate the mannose 6-phosphate residues on lysosomal enzymes (Ullrich *et al.*, 1978; Diment and Dean, 1983; Talkad and Sly, 1983). Treatment of the brain lysosomal enzymes with alkaline phosphatase did not in any way affect their binding to the receptor-Sepharose column and all the 4 enzymes completely bound as in the phosphatase untreated control experiment. The efficacy of the alkaline phosphatase was checked by measuring the bound phosphate in the Con A–Sepharose eluate before alkaline phosphatase treatment (5·47 μmol P_i per mg protein) and after alkaline phosphatase treatment (0·62 μmol P_i per mg protein) indicating almost 90% removal of the bound phosphate by the phosphatase.

To find out whether phosphatase treatment affects the affinity of the enzymes to the receptor protein, a gradient elution of the enzymes before and after phosphatase treatment by 0 → 35 mM mannose 6-phosphate followed by an elution at pH 4·5 was employed. The elution profile of β-glucosaminidase under such conditions is shown in figure 3. There were 3 peaks of the enzyme both before and after phosphatase treatment
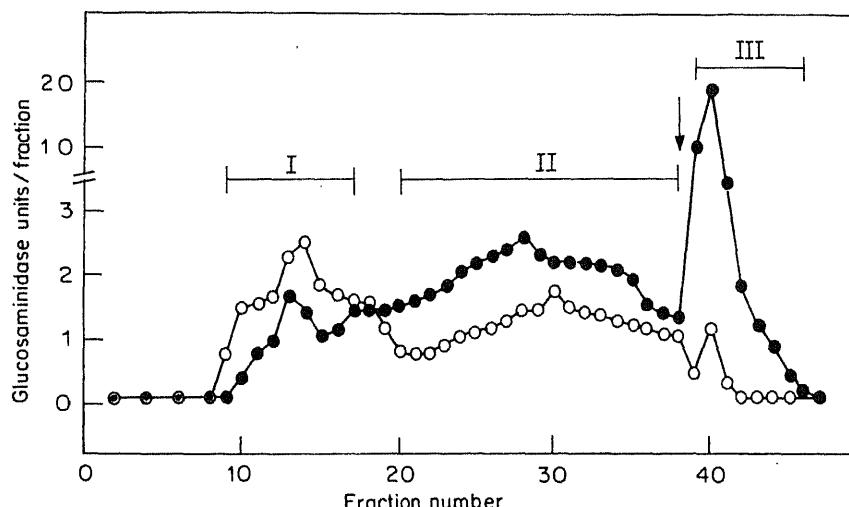
Figure 3. Elution profile of alkaline phosphatase treated (O) and untreated (●) β-glucosaminidase from the receptor–Sepharose column by a linear gradient (0→ 35 mM) of mannose 6-phosphate followed by citrate-phosphate buffer, pH 4·5. Con A–Sepharose eluate containing 150 units of β-glucosaminidase and an alkaline phosphatase treated Con A–Sepharose eluate containing 103 units of β-glucosaminidase were subjected to chromatography in separate experiments as given under 'materials and methods'. All the applied enzymes bound to the column completely. Elution with a gradient of mannose 6-phosphate was started from fraction 1 and ended at fraction number 38. Arrow indicates the start of elution with 20 mM citrate-phosphate buffer, pH 4·5. Recovery of enzyme activity from the column was 56% and 41% for the untreated and alkaline phosphatase treated enzymes respectively.

as shown in figure 3. Peaks I and II appeared during the mannose 6-phosphate gradient elution and peak III during the subsequent elution with 20 mM citrate-phosphate buffer, pH 4·5. The percentage of glucosaminidase eluted at low concentrations of mannose 6-phosphate (peak I) was only about 7 % of the eluted activity before alkaline phosphatase treatment but it increased to 45 % after phosphatase treatment with a significant decrease in peaks II and III (figure 3). These results suggested that a higher
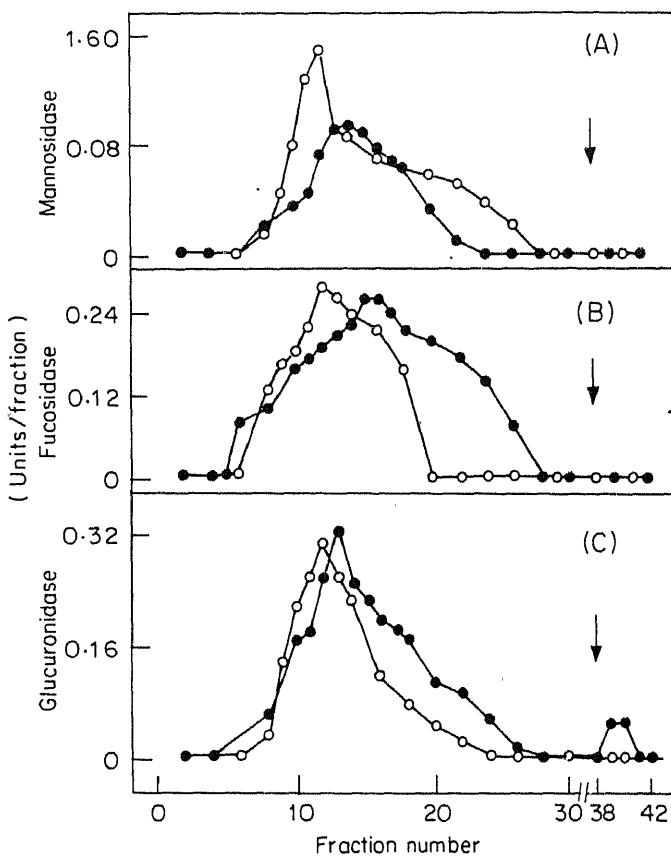
Figure 4. Elution profile of alkaline phosphatase treated (O) and untreated (●) mannosidase (A), fucosidase (B) and glucuronidase (C) from the receptor-Sepharose column by a linear gradient (0 → 35 mM) of mannose 6-phosphate followed by 20 mM citrate-phosphate buffer, pH 4·5.
   Con A–Sepharose eluate (containing 5 units of mannosidase, 19 units of fucosidase and 8 units of glucuronidase) and an alkaline phosphatase treated Con A–Sepharose eluate (containing 3·1 units of mannosidase, 15·2 units of fucosidase and 7·2 units of glucuronidase) were subjected to chromatography as given under 'materials and methods'. All the enzymes loaded bound to the column completely. Elution with a gradient of mannose 6-phosphate was started at fraction number 1 and ended at fraction number 38. Arrow indicates the start of elution with 20 mM citrate-phosphate buffer, pH 4·5. Recoveries of enzymes from the column were in the range of 22–30%, 30–35% and 14–20% for the enzymes mannosidase, glucuronidase and fucosidase, respectively.

concentration of mannose 6-phosphate and acid pH of 4·5 was needed for the elution of the major percentage of phosphatase untreated glucosaminidase whereas lower concentrations of mannose 6-phosphate could elute the major proportion of phosphatase treated glucosaminidase.

Figure 4 shows the elution profiles of mannosidase, fucosidase and glucuronidase during the gradient elution. These enzymes unlike the glucosaminidase emerged as single peaks. Prior treatment with alkaline phosphatase resulted in a shift of the elution peaks of all the 3 enzymes towards lower concentrations of mannose 6-phosphate. There was also less trailing of the enzymes after phosphatase treatment (figure 4).

Inability of serum pseudocholinesterase to bind to the receptor–Sepharose column

Serum pseudocholinesterase is a non-lysosomal glycoprotein capable of binding to Con A (George and Balasubramanian, 1981). When this enzyme was passed through the receptor–Sepharose column almost 92% of it was recovered unbound to the column and there was no detectable activity on elution of the column at pH 4·5.

Modifications of amino acids

Modifications of the amino acids residues of brain lysosomal enzymes by treatment with trinitro benzene sulphonic acid (for lysine, cysteine), phenylglyoxal (for arginine), N-acetyl imidazole (for tyrosine) and 2-hydroxy-5-nitro benzyl bromide (for tryptophan) as described under methods did not affect their binding to the receptor protein–Sepharose column (data not presented).

Discussion

It is possible that the lysosomal enzymes prepared from monkey brain used in the present studies is a mixture of phosphorylated and dephosphorylated forms (dephosphorylation of the mannose 6-phosphate residue occurs within the lysosomes) and also those containing phosphodiester residues. The almost complete abolition of binding to the receptor protein-Sepharose column by periodate treatment of the lysosomal enzymes clearly suggests that carbohydrate residues are essential for their binding. But the inability of phosphatase treatment to abolish or reduce the binding of any of the lysosomal enzymes to the column suggests that the phosphate moiety in mannose 6-phosphate is not absolutely essential for binding of the enzymes and that the brain receptor protein may recognise mannose or 'blocked' phosphate residues. These results are similar to the recent findings of Freeze (1985) on the interaction of alkaline phosphatase treated *D. discoideum* lysosomal enzymes with the immobilised phosphomannosyl receptor. This is also reflected in the ability of mannose and N-acetylglucosamine to inhibit the binding of the lysosomal enzymes to the soluble brain receptor reported earlier (Alvares and Balasubramanian, 1983). The brain receptor therefore appears analogous to the receptors in macrophages (Diment and Dean, 1983) and liver nonparenchymal cells (Ullrich *et al.*, 1979) which recognise mannose and N-acetylglucosamine. It is also quite possible that more than one type of receptor is involved in the binding of the enzymes as shown in macrophages (Shepherd *et al.*, 1984). These observations are also relevant to the findings of normal levels of lysosomal

enzymes in the brain and other organs of patients suffering from I-cell disease (Waheed *et al.*, 1982; Owada and Neufeld, 1982).

It is not clear why mannose and N-acetylglucosamine while inhibiting binding to the receptor protein (Alvares and Balasubramanian, 1983) failed to release the enzymes from the receptor–Sepharose column. One possibility is that even though the brain receptor can recognise mannose or N-acetylglucosamine in the absence of mannose 6-phosphate, the latter is still the better recognition marker and the one which is the 'best fit' to the binding site and so the only one potent enough to reverse the binding. Another possibility is that mannose and N-acetylglucosamine alone have too low an affinity to the receptor to be able to reverse the binding, but due to the large amounts of mannose and/or 'blocking' N-acetylglucosamine present on the enzyme molecule the residues interact co-operatively to provide the multivalent ligands with an affinity great enough to bind to the receptor (Talkad and Sly, 1983).

Results on the elution pattern of the lysosomal enzymes before and after phosphatase treatment from the receptor column by a gradient of mannose 6-phosphate show that after phosphatase treatment the major proportion of the enzymes could be eluted at lower concentrations of mannose 6-phosphate. This is more apparent in the case of β-glucosaminidase (figure 3). It is presumable that peak I of glucosaminidase is a dephosphorylated or less phosphorylated form of enzyme as compared to peaks II and III. These results lend support to the suggestion that although removal of phosphate from the lysosomal enzymes does not prevent their binding to the receptor column, it decreases the affinity with which they bind to the column. Although an 'uncovered' phosphate is not essential for binding the presence of phosphate is able to enhance the binding affinity.

The experiment showing that pseudocholinesterase, a non-lysosomal glycoprotein which binds to Con A does not bind to the receptor–Sepharose column rules out the possibility that the brain receptor used in this study behaves as a lectin that recognises mannose residues on glycoproteins in general. Conversely it strengthens the suggestion that the receptor isolated from brain is specific for lysosomal enzymes and can bind lysosomal enzymes through mannose 6-phosphate, mannose or 'blocked' phosphate residues although the mannose 6-phosphate recognition marker appears to be the one with higher affinity.

Acknowledgement

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Effect of mandur bhasma on lipolytic activities of liver, kidney and adipose tissue of albino rat during CCl₄ induced hepatic injury

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Abstract. 'Mandur bhasma', an ayurvedic preparation of iron is used in traditional medicine against hepatitis. In the present study the hepatoprotective property of this drug was tested in albino rats during CCl₄ induced hepatic injury. The effect of mandur bhasma on the activities of the lipolytic enzymes of rat liver, kidney and adipose tissue were studied during hepatitis induced by CCl₄. The activities of acid lipase, alkaline lipase, lipoprotein lipase and hormone sensitive lipase exhibited significant alterations during CCl₄ induced hepatic injury, indicating a role for these enzymes in the mobilization of fat from adipose tissue and accumulation of fat in liver and kidney. Simultaneous treatment with mandur bhasma prevented the paraffin mediated and CCl₄ mediated changes in the enzyme activities. These results suggest the hepatoprotective role of mandur bhasma during CCl₄ induced hepatic injury.

Keywords. Acid lipase; alkaline lipase; hormone sensitive lipase; lipoprotein lipase; mandur bhasma; liver; kidney; adipose tissue; CCl₄.

Introduction

Lipolytic enzymes play a very important role in the biological turnover of lipids and various forms of lipases have been reported (Brockerhoff and Jensen, 1974). Lipoprotein lipases have been detected in many tissues such as adipose tissue, mammary tissue, muscle, heart, aorta, liver, kidney, lung, spleen, medulla, diaphragm and fluids (plasma and milk) (Desnuelle, 1972; Fredrickson and Levy, 1972).

Hepatic triglyceride lipases have been assayed in liver homogenates (Varinkova and Mosinger, 1965), plasma membrane, cytosol, microsomes and lysosomes (Hayashi and Tappel, 1970; Assmann *et al.*, 1973; Teng and Kaplan, 1974; Debeer *et al.*, 1979). The cellular fractions show alkaline pH optima, except the lysosomal preparation which has an optimum pH of 4–6.

Mahadevan and Tappel (1968) reported the lysosomal and microsomal lipases of rat kidney. Similarly, Matsumura *et al.* (1976) reported 3 different forms of lipases in rat adipose tissue *viz.* lipoprotein lipase, hormone sensitive lipase and triglyceride lipase with alkaline pH optima. In our laboratory we have detected the lipolytic activity in rat adipose tissue having an acidic pH optimum of 4·2 (unpublished data). It is well known that treatment of CCl₄ to rats causes centrolobular hepatic necrosis leading to the accumulation of fat in liver and kidney. It was suggested that fats from the peripheral

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adipose tissue are translocated to the liver and kidney leading to accumulation during toxicity (Roullier, 1963).

Mandur bhasma, an ayurvedic preparation of iron (prepared using complex ayurvedic processes like Shodhana, Marana etc.) has long been used in the treatment of liver diseases (Sharma, 1977), but detailed biochemical studies are not available. Therefore, in the present investigation attempts have been made to find out the effect of mandur bhasma on lipolytic enzymes of liver, kidney and adipose tissue of albino rat during hepatitis induced by CCl_4.

Materials and methods

Swiss Norwegian male albino rats reared in the animal house of the department, were used for these experiments. The rats weighing 125–150 g were fed with standard laboratory diet (Hindustan Lever Ltd., Bombay) and water *ad libitum.*

Experimental protocol

Experiments were run in 5 sets and 5 rats were used in each group. To the first set, mandur bhasma (1 mg/100 g body wt.) was given orally for 11 days. To the second set paraffin (0·1 ml/100 g body wt.) was injected subcutaneously for 11 days. To the third set, paraffin (0·1 ml/100 g body wt.) was administered subcutaneously with the simultaneous oral administration of mandur bhasma (1 mg/100 g body wt.) for 11 days. To the fourth set 0·3 ml CCl_4 in liquid paraffin (3 : 1, v/v) per 100 g body wt. was given subcutaneously for 11 days. To the fifth set 0·3 ml CCl_4 in liquid paraffin (3 : 1, v/v) per 100 g body wt. was given subcutaneously concomitant with the oral administration of mandur bhasma (1 mg/100 g body wt.) for 11 days. The dose of mandur bhasma (1 mg/100 g body wt.) was found to be significantly protective in preliminary experiments. A group of 5 untreated rats was designated as normal.

Livers of the experimental animals were tested by routine biopsy methods on the 5th and 11th day to check for hepatic necrosis. Acute necrosis was observed on the 11th day in the livers of CCl_4 treated rats. All the animals were sacrificed on the 12th day by a sharp occipital blow. Liver, kidney and adipose tissue were dissected out and used for the assay of lipolytic activities. Hormone sensitive lipase, lipoprotein lipase and alkaline lipase were assayed according to Matsumura *et al.* (1976) using triolein as substrate, while the acid lipase activity was determined by the method of Mahadevan and Tappel (1968). At the end of incubation the enzyme activities were arrested and the liberated free fatty acids were estimated as described earlier (Patil *et al.*, 1983). Protein estimations were carried out in kidney and liver (Lowry *et al.*, 1951) and adipose tissue (Tornqvist and Belfrage, 1976) and lipid peroxidation was studied by the thiobarbituric acid method (Buege and Aust, 1978).

Results

Table 1 shows the changes in wet weights of liver, adipose tissue and kidney of normal, mandur bhasma, paraffin, paraffin + mandur bhasma, CCl_4 and CCl_4 + mandur

Table 1. Alterations in fresh weights of different tissues of rats treated with mandur bhasma, paraffin, paraffin + mandur bhasma, CCl_4 and CCl_4 + mandur bhasma.

Treatment	Liver	Adipose tissue	Kidney
Normal	3.836 ± 0.145	0.675 ± 0.036	0.634 ± 0.043
Mandur bhasma	3.460 ± 0.155^b	0.397 ± 0.026^a	0.525 ± 0.029^d
Paraffin	3.462 ± 0.160^b	0.637 ± 0.035^b	0.693 ± 0.033^a
Paraffin + mandur bhasma	3.325 ± 0.146^b	0.358 ± 0.021^a	0.471 ± 0.035^a
CCl_4	4.224 ± 0.233^a	0.510 ± 0.027^a	0.707 ± 0.037^a
CCl_4 + mandur bhasma	3.842 ± 0.187^b	0.670 ± 0.037^b	0.658 ± 0.031^d

Values are expressed as g/100 g body wt.
$^a P < 0.05$; $^b P > 0.05$ compared to the control group.

bhasma treated rats. Treatment of mandur bhasma, paraffin and paraffin + mandur bhasma reduced the weights of liver, kidney and adipose tissue, paraffin treated rat kidney being the exception. A marked fall in the wet weights of kidney and adipose tissue of mandur bhasma treated rats was noticed as compared to that of normal rats, while the treatment with CCl_4 caused an increase in the wet weights of liver and kidney with a decrease in the wet weight of adipose tissue. Simultaneous treatment of CCl_4 and mandur bhasma did not exhibit the marked changes observed in the wet weights of these tissues.

Histologically the liver of mandur bhasma treated rats showed absolutely normal appearance having very rare binucleated cells. Paraffin treatment caused an increase in the size of hepatic cells which were stained intensely with eosin and the nuclei did not show staining with hematoxylin although they showed positive Feulgen reaction. Treatment of mandur bhasma concomitant with paraffin exhibited the normal histological picture of the liver. The blood in the blood vessels of the liver, which was vacuolated in paraffin treated rat became normal by treatment of mandur bhasma along with paraffin. CCl_4 treatment showed hepatic necrosis which was evidenced by the detection of highly vacuolated cells. Though the simultaneous treatment of mandur bhasma and CCl_4 did not alter the histological picture of the liver significantly, it showed an increased number of binucleated cells and mitotic figures.

The kidney of mandur bhasma treated rats displayed the normal histological picture. On the contrary paraffin treatment showed foggy patches of tubules mainly in the cortex region with the vacuolar appearance of blood in blood vessels, but treatment of mandur bhasma along with paraffin demonstrated the normal appearance of the kidney with a conspicuous decrease in the fogginess of tubules. The appearance of the blood in blood vessels was normal. Similarly the conspicuous foggy necrosis of proximal tubules observed in the kidney of CCl_4 treated rats was significantly reduced in CCl_4 + mandur bhasma treated rats (data not presented).

Alterations in the lipolytic activities of the liver of normal rats and the rats treated with mandur bhasma, paraffin, paraffin + mandur bhasma, CCl_4 and CCl_4 + mandur bhasma are represented in table 2. Mandur bhasma, paraffin and paraffin + mandur bhasma treatments caused an increase in alkaline and lipoprotein lipase activities, while

Table 2. Effect of administration of mandur bhasma, paraffin, paraffin + mandur bhasma, CCl_4 and CCl_4 + mandur bhasma on the lipolytic activities of rat liver.

Treatment	Lipolytic activities					
	Acid lipase		Alkaline lipase		Lipoprotein lipase	
	K units/g wet wt.	Units/mg protein	K units/g wet wt.	Units/mg protein	K units/g wet wt.	Units/mg protein
Normal	10·00 ± 0·59	33·33 ± 2·33	2·00 ± 0·11	6·67 ± 0·31	6·00 ± 0·26	20·00 ± 0·86
Mandur bhasma	1·40 ± 0·06[c]	5·28 ± 0·21[c]	6·40 ± 0·28[b]	24·15 ± 1·13[c]	7·00 ± 0·09[d]	26·41 ± 0·99[b]
Paraffin	7·20 ± 0·48[a]	31·30 ± 1·59[b]	7·86 ± 0·41[b]	34·17 ± 1·48[c]	7·80 ± 0·47[a]	33·91 ± 1·30[b]
Paraffin + mandur bhasma	23·60 ± 0·91[c]	118·00 ± 3·75[c]	7·80 ± 0·41[b]	39·00 ± 1·33[c]	6·20 ± 0·46[d]	31·00 ± 1·52[b]
CCl_4	3·84 ± 0·18[c]	16·70 ± 0·61[b]	12·50 ± 0·46[c]	54·35 ± 2·75[a]	2·98 ± 0·12[b]	12·78 ± 0·44[a]
CCl_4 + mandur bhasma	7·20 ± 0·56[a]	38·91 ± 1·88[a]	15·60 ± 0·91[c]	84·31 ± 3·16[b]	9·46 ± 0·85[a]	51·13 ± 2·03[c]

Values are mean ± S. E. of 5 animals.
[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$; [d]$P > 0.05$ compared to the control group.

these treatments lowered the acid lipase activity but with a significant increase in acid lipase activity of paraffin + mandur bhasma treated rat liver. Lysosomal and lipoprotein lipase activities of CCl_4 treated rat liver showed a decrease, while the alkaline lipase activity of CCl_4 treated rats showed an increase compared to normal rats. The enzyme specific activities expressed per mg protein paralleled the changes in the activities expressed per gram wet weight of liver indicating the alterations in enzyme proteins *per se*. Mandur bhasma treatment along with CCl_4 resulted in an increase in the activities of all the enzymes compared to the levels obtained with CCl_4 or mandur bhasma treatment only. Concomitant treatment of mandur bhasma and paraffin did not alter the enzyme activities, except the lysosomal lipase which showed an increase compared to the levels of mandur bhasma and paraffin treated rats.

Table 3 shows the changes in the lipolytic activities of adipose tissue under the given experimental conditions. Mandur bhasma treatment resulted in the sharp fall in the activities of hormone sensitive, alkaline and lipoprotein lipases. The treatment of paraffin caused the conspicuous rise in the activities of all the 4 lipases. Mandur bhasma given concomitant with paraffin resulted in the marked decrease in enzyme activities compared to normal rats, but resembled the enzyme activities of mandur bhasma treated rat adipose tissue. Treatment of mandur bhasma alone did not alter the acid lipase activity significantly. Similarly, treatment of mandur bhasma along with paraffin showed full protection to acid lipase activity. A significant increase in the activities of all lipolytic enzymes was observed in CCl_4 treated rats, except in the case of lipoprotein lipase, which showed a decrease. Mandur bhasma treatment prevented the CCl_4 mediated increase in acid and hormone sensitive lipase activities. It also counteracted the decrease in lipoprotein lipase activity. Mandur bhasma had a marginal effect on the CCl_4 mediated increase in alkaline lipase activity. The alkaline lipase activity of CCl_4 + mandur bhasma treated rat adipose tissue exhibited a marginal increase when expressed per mg protein, but exhibited a marginal decrease when expressed per gram fresh tissue. Simultaneous treatment of mandur bhasma and CCl_4 resulted in the sharp increase in the activities of all lipases. Parallel variations were noticed when activities were expressed per gram fresh weight and per mg protein.

Variations in the lipolytic activities of the kidney of normal rats and the rats treated with mandur bhasma, paraffin, paraffin + mandur bhasma, CCl_4 and CCl_4 + mandur bhasma are given in table 4. The enzyme activities declined in the paraffin treated rats as well as CCl_4 treated groups compared to normal animals; but the reductions were more in the paraffin treated rats than in the CCl_4 treated rats. Treatment of only mandur bhasma and paraffin + mandur bhasma exhibited a conspicuous fall in all the enzyme activities compared to normal rats except in the case of lysosomal lipase activity in paraffin + mandur bhasma treated rats. Lysosomal and lipoprotein lipase activities of paraffin + mandur bhasma treated rats were significantly higher than in mandur bhasma treated rats, but alkaline lipase did not exhibit a significant change in its activity. Mandur bhasma counteracted the CCl_4 mediated decrease in the enzyme activities. The activities of all the enzymes of CCl_4 + mandur bhasma treated rats were conspicuously higher than in mandur bhasma or CCl_4 treated rats. Similar to liver and adipose tissue lipases, the enzymes of kidney also exhibited the parallel alterations in enzyme activities when expressed per gram fresh weight and per mg protein indicating changes in enzyme proteins *per se*.

Table 3. Effect of administration of mandur bhasma, paraffin, paraffin + mandur bhasma, CCl_4 and CCl_4 + mandur bhasma on the lipolytic activities of rat adipose tissue.

Treatment	Acid lipase		Hormone sensitive lipase		Alkaline lipase		Lipoprotein lipase	
	K units/g wet wt.	Units/mg protein	K units/g wet wt.	Units/mg protein	K units/g wet wt.	Units/mg protein	K units/g wet wt.	Units/mg protein
Normal	1·96±0·08	9·80±0·46	8·99±0·32	44·95±1·94	16·18±0·70	80·90±3·85	8·34±0·67	41·70±2·34
Mandur bhasma	2·17±0·13[d]	42·35±3·23[c]	0·16±0·01[c]	3·26±0·13[c]	0·31±0·06[c]	6·13±0·21[c]	0·66±0·03[c]	13·02±0·85[b]
Paraffin	27·57±1·19[c]	119·87±4·43[c]	27·51±2·30[c]	119·61±5·11[c]	17·81±0·93[d]	77·39±4·07[d]	17·82±1·22[b]	77·48±3·62[b]
Paraffin + mandur bhasma	2·25±0·09[d]	48·14±3·11[c]	0·24±0·02[c]	5·26±0·22[c]	0·12±0·02[c]	2·63±0·11[c]	0·37±0·02[c]	7·90±0·36[c]
CCl_4	60·82±2·43[c]	202·73±6·53[c]	47·96±3·22[c]	159·87±6·14[c]	31·76±1·84[b]	105·67±4·82[a]	1·05±0·02[c]	3·50±0·31[c]
CCl_4 + mandur bhasma	7·13±0·37[f]	31·01±5·71[c]	15·95±0·83[b]	69·31±2·77[a]	28·22±0·05[b]	127·70±5·92[b]	13·10±1·06[a]	56·44±2·86[a]

Values are mean ± S.E. of 5 animals.   P values are as in table 2.

Table 4. Effect of administration of mandur bhasma, paraffin, paraffin + mandur bhasma, CCl_4 and CCl_4 + mandur bhasma on the lipolytic activities of rat kidney.

	Lipolytic activities					
Treatment	Acid lipase		Alkaline lipase		Lipoprotein lipase	
	K units/g wet wt.	Units/mg protein	K units/g wet wt.	Units/mg protein	K units/g wet wt.	Units/mg protein
Normal	13·60±0·57	68·00±4·50	13·60±0·78	68·00±4·13	12·00±0·76	60·00±3·82
Mandur bhasma	4·44±0·21[c]	33·85±1·20[b]	1·60±0·06[c]	12·31±0·55[c]	1·24±0·05[c]	9·54±0·38[c]
Paraffin	3·76±0·13[c]	18·80±0·75[c]	10·20±0·52[a]	51·00±2·05[a]	0·90±0·06[c]	4·50±0·22[c]
Paraffin + mandur bhasma	21·00±0·75[b]	91·30±3·68[b]	1·65±0·06[c]	7·17±0·19[c]	4·40±0·17[b]	19·13±0·73[b]
CCl_4 treated	5·40±0·21[c]	29·19±1·61[b]	10·40±0·66[a]	56·22±3·14[a]	1·80±0·08[c]	9·73±0·51[c]
CCl_4 + mandur bhasma	14·46±0·81[d]	72·30±4·90[a]	34·80±1·83[c]	174·00±5·53[c]	6·40±0·35[b]	32·00±1·27[b]

Values are mean ± S.E. of 5 animals.   P values are as in table 2.

Discussion

The treatment of mandur bhasma resulted in a decrease in lysosomal lipase of liver with increase in lipoprotein lipase and alkaline lipases, which suggest the increased secretion of lipoproteins as well as release of fatty acids needed for the metabolic processes, because the histological architecture of liver was totally normal and there was no detectable lipid peroxidation. The lowered lipolytic activities of adipose tissue and kidney due to mandur bhasma treatment, except in the case of acid lipase of adipose tissue indicate the reduced uptake and mobilization of lipids. The mass of adipose tissue of mandur bhasma treated rats was decreased by about 41 % than normal rats, but the lipids of adipose tissue of mandur bhasma treated rats (877 mg/g fresh wt.) was not altered significantly compared to normal rats (918 mg/g fresh wt.). The reduction in the mass of adipose tissue without alteration in lipid content along with the increased acid lipase activity by mandur bhasma is a clear indication of adipose tissue lysis. These observations lend credence for the use of preparations of iron such as mandur bhasma, Louh bhasma in the treatment of obesity (Sharma, 1977).

Similar lines of argument can be advanced to suggest that the paraffin toxicity results in a high turnover of lipids in the adipose tissue and that mandur bhasma treatment counteracts this situation. In the liver, mandur bhasma may bring about the enhanced release of fatty acids to protect against paraffin toxicity. In the kidney, this protection may involve enhanced uptake of lipids and elevated lysosomal lipolysis.

Mandur bhasma in general counteracted the effects of CCl$_4$ on the levels of lipases. The higher liver lipoprotein lipase activity in CCl$_4$ + mandur bhasma treated rats than in CCl$_4$ treated rats suggests the increased secretion of liver lipoproteins and rapid uptake of fatty acids by adipose tissue. The acid and hormone sensitive lipolytic activities in the adipose tissue were significantly lowered in mandur bhasma + CCl$_4$ treated rats. From these observations it appears that the rate of lipolysis was less than the uptake of fatty acids by adipose tissue in CCl$_4$ + mandur bhasma treated rats. Mandur bhasma also counteracted the decrease in liver and kidney lysosomal lipase activities brought about by CCl$_4$ treatment. Surprisingly alkaline lipase activities of all the 3 tissues in CCl$_4$ + mandur bhasma treated rats were significantly higher than those observed in normal and only mandur bhasma or CCl$_4$ treated rats. When the liver homogenate of CCl$_4$ treated rats was incubated in the presence of mandur bhasma, the rate of lipid peroxidation was significantly lower (3·33 %) when compared with the lipid peroxidation in the absence of mandur bhasma (100 %). It is possible that the increased alkaline lipase in CCl$_4$ + mandur bhasma treated rats is responsible for the synthesis of complex lipids of membranes and other cellular components for the regeneration of new cells in these tissues. Histologically, the liver and kidneys were not damaged significantly and new regenerating hepatic regions were observed in the region of necrosis. Similarly lysosomal lipase activities of liver and kidney of CCl$_4$ + mandur bhasma treated rats were significantly higher than in rats treated with mandur bhasma only. This increase may be attributed to the enhanced release of fatty acids to meet the metabolic energy demand during the protection of the liver and kidney by mandur bhasma against CCl$_4$ induced toxicity.

Further studies are being carried out in this laboratory on the chemical nature of mandur bhasma and on the mechanism of its hepatoprotective action in hepatopathology.

Acknowledgement

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Cross reactivity and enzyme sensitivity of immunoaffinity purified Sm/RNP antigens

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Abstract. Immunoaffinity purified Sm/RNP antigens from buffalo and goat liver were studied to determine the role of RNA and proteins towards the antigenicity of Sm and RNP antigens. A more direct approach using enzyme-linked immunosorbent assay on nylon beads has been utilized to look into the problem. The effect of enzyme treatment and the role of RNA and protein fractions in influencing antigenicity have been described. RNA seems to be involved in the maintenance of RNP specific polypeptides in suitable conformation so as to keep them in solution. Removal of RNA leads to insolubilization of RNP specific polypeptides. Antibodies to Sm and RNP antigens have been shown to cross react with poly A containing heterogeneous nuclear ribonucleoprotein with no cross reactivity with thymus RNA or DNA.

Keywords. Sm/RNP; hnRNP; autoantibodies; enzyme-linked immunosorbent assay; enzyme sensitivity.

Introduction

Autoantibodies produced by patients with autoimmune diseases have been found to be useful tool for the characterization of small nuclear ribonucleoprotein (snRNP) complexes (Lerner and Steitz, 1979). These highly conserved RNA-protein complexes, which are targets of autoimmunity have been classified as Sm and RNP antigens on the basis of differential sensitivity to RNase (Mattioli and Reichlin, 1971; Northway and Tan, 1972; Ishaq and Ali, 1983a). Recently, the two antigens have been differentiated and characterized on the basis of the type and number of their antigenic polypeptides (Hinterberger *et al.*, 1983; Ishaq and Ali, 1983b; Steitz *et al.*, 1983). Role of RNA and the exact number and size of polypeptides responsible for the antigenic activity however, remains controversial. Further, the specificity and cross reactivity of the antibodies reactive with Sm and RNP antigens remains to be examined.

Abbreviations used: RNP, Ribonucleoprotein; snRNP, small nuclear RNP; hnRNP, heterogeneous nuclear RNP; SLE, systemic lupus erythematosus; DEAE, diethylaminoethyl; PMSF, phenylmethylsulphonyl fluoride; IgG, immunoglobulin-G; PBS, phosphate buffer saline; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; UV, ultraviolet.

The present study describes the isolation and characterization of Sm/RNP antigens from buffalo and goat liver. The immunoaffinity purified antigenic particles were subjected to characterization with an attempt to check the cross reactivity of antibodies with other antigens including hnRNP particles which are RNA-protein complexes residing with Sm/RNP particles in the same nuclear envelope.

Materials and methods

Isolation and purification of Sm/RNP antigen

Sm/RNP antigenic particles were isolated from fresh buffalo and goat liver as described earlier (Ishaq and Ali, 1983b). Nuclei were isolated (Douvas *et al.*, 1979) and extracted with STM buffer pH 8·0 (10 mM Tris-HCl, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride (PMSF), 100 μg/ml polyvinyl sulphate). The insoluble material was removed by centrifugation and the supernatant passed successively through Sepharose CL-2B and diethylaminoethyl (DEAE) cellulose columns (Ishaq and Ali, 1984). During purification, only those fractions containing RNA and protein and Sm/RNP activity were pooled and processed further. Ion exchange purified material was applied to an affinity column of immunoglobulin-G (IgG) of a Sm/RNP positive systemic lupus erythematosus (SLE) serum linked to CNBr-activated Sepharose 4B. The eluate after extensive dialysis was passed through another affinity column prepared by linking normal human IgG to Sepharose 4B. The column was washed with phosphate buffer saline (PBS) till no protein was eluted. Eluate from normal IgG-Sepharose column was used as purified Sm/RNP antigen.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) for Sm/RNP antigen was carried out with affinity purified antigen using nylon beads as described earlier (Ishaq and Ali, 1983a). ELISA for antibodies against RNA and DNA was performed as already reported (Kumar and Ali, 1984a; Ali and Ali, 1986). Sera tested for anti-RNA antibodies were pretreated with aurintricarboxylic acid polymer to overcome serum RNase activity (Kumar and Ali, 1984b).

Treatment of antigen with immobilized RNase and trypsin

RNase A and trypsin (Sigma Chemical Co., USA) were linked to CNBr-activated Sepharose 4B as per instructions of the manufacturer. Immunoaffinity purified RNP was digested for different time intervals individually with immobilized RNase and trypsin. At the end of incubation, the immobilized enzymes were separated by centrifugation in cold. The resulting soluble antigen was coated on nylon beads and processed for ELISA using Sm and RNP reference sera.

Dissociation of Sm/RNP particles

Affinity purified antigen was dissociated into protein and RNA fractions by a modified procedure of White *et al.* (1982). Antigen was dialysed against PBS containing 8 M urea,

2 mM 2-mercaptoethanol and 0.1 mM PMSF. The dialysed material was passed through the DEAE cellulose column equilibrated with the same buffer. Proteins eluted in the wash volume were collected and dialysed against PBS. RNA bound to the column was eluted with 10 mM P_i, pH 7·2 containing 3 M NaCl. The isolated RNA was extensively dialysed against PBS.

Spectral studies

Ultraviolet (UV) spectra of Sm/RNP particles, dissociated RNA and protein fractions were recorded in PBS, pH 7·2 using Spectronic-21 spectrophotometer.

Carbohydrate estimation

Carbohydrate content of affinity purified antigen was estimated by the method of Winzler (1955).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Polypeptide analysis of Sm/RNP antigens was performed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). Samples for electrophoresis were solubilized in 10 mM Tris-HCl, pH 8·8 containing 1 % SDS and 5 % 2-mercaptoethanol and heated for 5 min at 90°C. Fifty μg of protein was applied to 10 % polyacrylamide gel slab containing 0·1 % SDS and electrophoresed at 10 mA and 50 volts for 20 h as described by Laemmli (1970). Phosphorylase B (97,400 d), BSA (68,000 d), ovalbumin (43,000 d), carbonic anhydrase (25,000 d) and cytochrome C (11,700 d) were used as molecular weight markers. At the end of electrophoresis, the slab was fixed for 1 h in 25 % isopropanol and 10 % acetic acid. Staining was carried out for 20 min in 0·2 % Coomassie brilliant blue R-250 in 50 % methanol and 10 % acetic acid. The slab was destained by 25 % methanol plus 10 % acetic acid.

Isolation of poly A-hnRNP particles by oligo-dT cellulose column chromatography

Oligo-dT cellulose (Sigma Chemical Co., USA) was treated with 0·1 M NaOH for 10 min, extensively washed with distilled water and equilibrated with binding buffer (10 mM P_i, pH 7·0, 0·25 M NaCl). Nuclear extract was dialysed against binding buffer and loaded onto the oligo-dT cellulose column. The column was washed with copious volumes of buffer to remove unbound material. The bound material was eluted with 10 mM P_i, pH 7·0 having 50 % deionized formamide. Poly A-hnRNP thus eluted was dialysed against PBS and hybridized with an excess of poly U in PBS pH 7·2 and rechromatographed on oligo-dT cellulose. Ninety five per cent of the loaded material remained unbound and was eluted in wash.

Thymus RNA and DNA

RNA from buffalo thymus was isolated as described elsewhere (Kumar *et al.*, 1983). DNA obtained from Sigma Chemical Co., St. Louis, Missouri, USA, was purified by hydroxyapatite column chromatography (Ali and Ali, 1984). Double-stranded DNA was heat denatured to get single stranded DNA. Normal human sera were obtained

from healthy subjects. Sera of patients with SLE were collected as described earlier (Ishaq *et al.*, 1982). Serum IgG was isolated by ion-exchange chromatography using DEAE cellulose (Ali *et al.*, 1983).

Results

Isolation of Sm/RNP antigen was carried out under RNase free conditions. The RNA:protein ratio of the affinity purified material was 5·2:1 by weight with 27% carbohydrate content. The 280/260 ratio of the glycoprotein RNP complex was 1·10. The UV spectra of the Sm/RNP complex is depicted in figure 1. The affinity purified material was antigenically active when tested by ELISA and showed strong antibody activity with Sm, RNP and Sm/RNP reference sera.
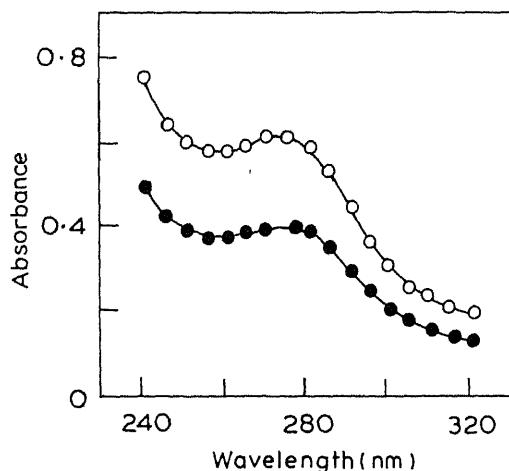
Figure 1. UV spectra of purified Sm/RNP antigen (O) and protein (●) fraction.

The polypeptide analysis of the antigen from buffalo and goat liver at different stages of purification was carried out by SDS-PAGE (data not given). Exactly similar profiles were observed with antigens isolated from two different sources. Five major polypeptides of molecular weight 80K, 70K, 29K, 13K and 12K were obtained from affinity purified antigen.

After dissociation and separation of RNA and protein parts of Sm/RNP antigens and upon removal of urea, a substantial precipitation occurs in the protein fraction. The UV spectral characteristics of the soluble protein fraction is given in figure 1. The soluble fraction was tested for Sm and RNP antigenicity using Sm and RNP reference

sera. Ninety per cent of activity was consistently obtained as Sm-activity in the soluble fraction with Sm reference sera as compared to 8–10% of RNP activity (figure 2). No significant antibody activity was present in the RNA fraction with RNP, Sm and Sm/RNP reference sera (data not shown).
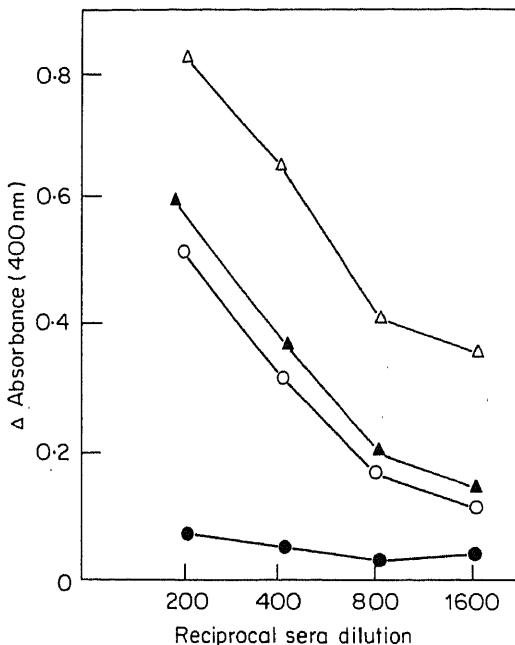
Figure 2. Antigenicity of the soluble protein fraction obtained by dissociation of RNP complex.
RNP complex was dissociated into RNA and protein fractions as described in 'materials and methods'. Soluble protein fraction (100 μg/ml) was coated on nylon beads and tested by ELISA using anti-Sm (O) and anti-RNP (●) reference sera. Undissociated Sm/RNP coated beads tested with Sm (▲) and RNP (Δ) reference sera.

RNP antigenicity was lost on incubation with the immobilized RNase when tested with RNP reference serum. The loss in antibody activity was dependent on the period of incubation with the enzyme. Sm activity, on the other hand, remained almost unaffected by RNase treatment. Trypsin treatment resulted in the loss of both Sm and RNP antigenic activity (figure 3). The specificity and cross reactivity of Sm/RNP antibodies were checked by ELISA using RNA, ssDNA, dsDNA and poly A containing hnRNP. No cross reactivity was observed when Sm/RNP reference sera were tested for reactivity with mammalian RNA and ds- or ssDNA (data not given). With poly A-hnRNP, around 22% reactivity was consistently observed with sera positive for antibodies against Sm/RNP antigens (figure 4).
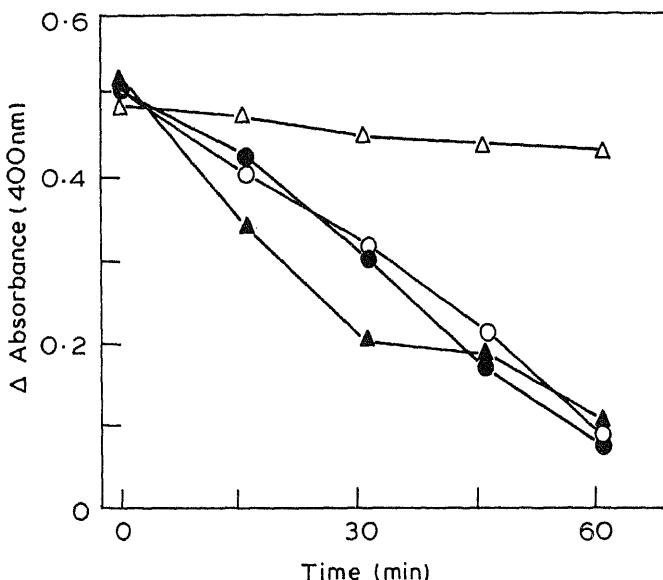
Figure 3. Enzyme sensitivity of Sm/RNP antigens.

Sm/RNP antigens were treated with immobilized trypsin and RNase for different time intervals. Trypsin treated antigen was coated on nylon beads and tested with anti-Sm (O) and anti-RNP (●) reference sera. Similarly, RNase treated antigen was coated on nylon beads and processed for ELISA using anti-Sm (Δ) and anti-RNP (▲) reference sera. Serum dilution was 1:200.
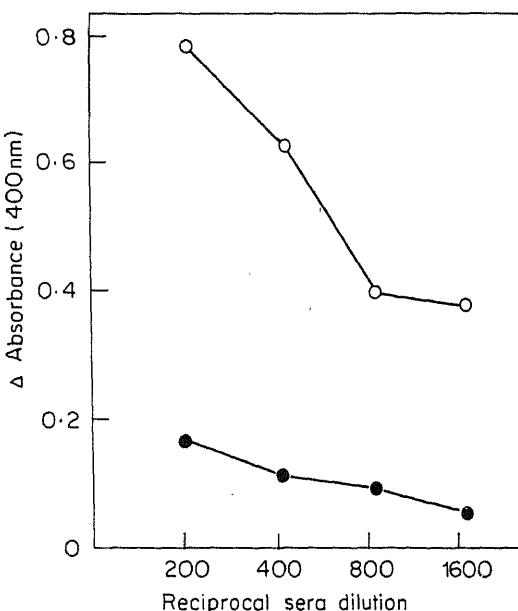
· **Figure 4.** Cross reactivity of anti-Sm/RNP antibodies with poly A containing hnRNP. ELISA was performed using affinity purified Sm/RNP (O) and poly A containing hnRNP (●) coated nylon beads at various dilutions of anti-Sm/RNP positive serum.

Discussion

Sm/RNP ribonucleoprotein antigens were isolated from buffalo and goat liver by immunoaffinity chromatography. The polypeptide profiles from two different sources were identical revealing no species difference and reiterating the belief that these molecular complexes are highly conserved molecules. When the nuclear extract was directly loaded onto the immunoaffinity column, the yield of the purified antigen was considerably low although, no difference was observed in the polypeptide profile (data not shown). Thus the purification could be shortened considerably permitting rapid isolation of the antigen provided yield is of secondary importance. The UV spectra of RNP complex, proteins:RNA ratio and carbohydrate content of the preparation reveals that the antigen is a glycoprotein-RNA complex. There has been considerable variation in the reported molecular weight values of antigenic polypeptides associated with Sm and RNP antigens. The demonstration of 5 major polypeptides of molecular weight 80K, 70K, 29K, 13K and 12K in this study is in good agreement with those found by other investigators in preparations from other sources (White *et al.*, 1981; Lerner and Steitz, 1979; Billings *et al.*, 1982). A similar number of polypeptides was reported from this laboratory in an earlier study (Ishaq and Ali, 1983b).

The antigenicity of purified RNP requires an intact RNA for antibody activity. RNase treatment of antigen in crude or purified form, destroys the immunological determinant for RNP antibody whereas RNase-treated RNP does retain Sm reactivity (figure 3). It is generally believed that Sm determinant is defined by protein alone (Takano *et al.*, 1981; White *et al.*, 1982; Lerner *et al.*, 1981). To look into the role of RNA in antigenicity, a non-conventional procedure was adopted. The purified antigen having both Sm and RNP activities was subjected to the action of immobilized RNase for different time intervals. The resulting antigenic preparations were coated on nylon beads and ELISA was carried out using Sm and RNP reference sera. RNP antigenicity was gradually lost and within an hour of incubation with RNase, almost complete loss in antigenicity was observed. No significant change in the Sm activity was noticed. To further explore the requirement of RNA in RNP antigenicity, Sm/RNP complex was separated into RNA and protein fractions. The isolated RNA, free of protein contamination, was tested for its reactivity with Sm, RNP and Sm/RNP reference sera by ELISA. No antibody activity was found to be associated with RNA fraction of Sm/RNP antigen thereby implying that on its own, RNA has got no activity in Sm/RNP antigenicity. In a recent study a small portion of the entire RNA sequence and few peptides have been implicated as the RNP antigenic determinant in calf thymus preparation (Agris *et al.*, 1984).

During the separation of RNA and protein from Sm/RNP complex, it was observed that a significant part of the protein fraction becomes insoluble upon removal of urea. The soluble portion was coated on nylon beads and processed for ELISA. The antigen on the solid support was fully active towards Sm antibody and showed insignificant activity towards anti-RNP antibody. The insolubilization of these polypeptides was successfully reversed when RNA fraction was presented in solution at the time of removal of urea. The RNP antigenicity was also restored. This led us to believe that RNA maintains RNP polypeptides in solution and somehow expose the antigenic determinant in these complexes. Upon removal or degradation of RNA, the antigenic

polypeptides are rendered insoluble and it is inferred that the RNP activity is lost as a result of RNase treatment.

The close association of RNP complexes with poly A-hnRNP in the nucleus and possible association of some of the antigenic polypeptides in these particles is likely to explain their cross reactivity (figure 4). Sera from patients with mixed connective tissue disease were found to contain antibodies that react with at least two species of RNP, snRNP and a high molecular weight hnRNP/RNA bound to the nuclear matrix (Ali and Tan, 1979; Fritzler *et al.*, 1984). In another study, human autoimmune sera were found to interact with hnRNP and these autoantibodies recognized common epitopes present on snRNP and hnRNP (Zouali and Eyquem, 1984).

Acknowledgements

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Effects of piretanide on plasma fibrinolytic activity, platelet aggregation and platelet factor-4 release in man

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Abstract. Piretanide, 4-phenoxy-3-(pyrrolidinyl)-5-sulphamoyl benzoic acid, apart from being an efficient diuretic, enhances endogenous plasma fibrinolytic activity after a single dose of 6 mg administered by oral route. After ingestion of the drug, acceleration of fibrinolytic acitivity became manifest within 1 h, reached its peak in 3 h and was associated with a fall in fibrinogen and diminished urokinase excretion. Piretanide did not cause lysis of fibrin *in vitro*. Primary platelet aggregation, induced by adenosine-diphosphate, was inhibited by piretanide. In *in vitro* experiments piretanide led to effective inhibition of adenosine-diphosphate-induced platelet aggregation with complete inhibition at 5 mM concentration. Piretanide led to a highly significant decrease of platelet factor-4 release.

Keywords. Piretanide; synthetic fibrinolytic agent; anti-platelet agent; PF-4 release; diuretic.

Introduction

A great deal of interest is involved in search of newer synthetic agents with antithrombotic and fibrinolytic properties to exploit the natural system of thrombolysis and to gain long-term fibrinolytic prophylaxis against degenerative vascular disorders (Davidson and Walker, 1979). Several combinations of thrombolytic and anti-platelet agents have been tried, including heparin, plasminogen with streptokinase, urokinase and ancrod, to achieve better results by promoting fibrinolysis and inhibiting platelet aggregation and/or release reaction, with variable results and attendant immunological problems. However, the attraction of such combinations is that smaller doses of thrombolytic agents may be effective, thus reducing episodes of bleeding complications (Kwaan, 1979). In our experience, furosemide (Lasix®) effectively reverses the adverse changes in blood coagulation in high altitude pulmonary oedema which is associated with diminished fibrinolytic activity and hypercoagulability (Singh and Chohan, 1973, 1974). At present, smaller doses of 20 mg furosemide, administered orally or intravenously, are advocated to restore fibrinolytic activity and to achieve inhibition of platelet aggregation and release reaction in this disorder (Chohan, 1980, 1984). A close similarity in structure and function, between furosemide and piretanide, as diuretics, prompted the present study to elucidate effects of the latter on plasma

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Abbreviations used: PF-4, Platelet factor-4; ADP, adenosine-diphosphate; PRP, platelet rich plasma; PAT, platelet aggregation times; f.c., final concentration; PGG_2, prostaglandin endoperoxides.

fibrinolytic activity, platelet aggregation and release of platelet factor-4 (PF-4). Piretanide, 4-phenoxy-3-(1-pyrrolidinyl)-5-sulphamoyl benzoic acid, is a new diuretic whose effects are similar to those of furosemide in man and animals (Lawrence *et al.,* 1978) but its effects on blood coagulation are not known.

Materials and methods

Subjects, drug and reagents

Twenty two males with an average age of 25·8 ± 3·8 years were studied with their informed consent. Parameters estimated, before and at different intervals after ingestion of 6–12 mg piretanide included plasma euglobulin fibrinolytic activity (Astrup and Mullertz, 1952), urokinase activity (Chohan *et al.,* 1977a), fibrinogen contents (Ratnoff and Menzie, 1951), PF-4 availability (Harada and Zucker, 1971; Chohan *et al.,* 1977b) and platelet aggregation and platelet aggregation times (Born, 1964; Chohan *et al.,* 1977b). Piretanide (molecular weight, 362·4) in powder form was dissolved in 100 mM Tris buffer (pH 11·0) containing 95 mM NaCl and 3 mM KCl and pH was adjusted to 7·5 with HCl. A stock solution of 50 mM was diluted when desired for *in vitro* studies. All concentrations were expressed as final concentration (f.c.). Piretanide in powder as well as in tablet form, dispensed in 3–12 mg, was used (Farbwerke Hoechst, A. G., Frankfurt, Germany, designated as HOE-118).

Other reagents used in the study were Adenosine-diphosphate (Sigma Chemical Co., St. Louis, Missouri, USA), Fibrinogen (Poviet, N. V., Amsterdam, The Netherlands), Heparin (Liquemine, Roche, Basel, Switzerland) and Bovine Thrombin (Parke Davis, Detroit, Michigan, USA) as per directions of the manufacturers.

Fibrinolytic activity and fibrinogen

Thirteen subjects formed part of this study, who were administered 6 mg of piretanide orally. Euglobulin fibrinolytic activity was measured as areas of lysis in mm^2 on blood samples collected from them at intervals of 0 h to 1, 3, 6, 12 and 24 h. Simultaneously, plasma fibrinogen contents were determined in 9 subjects.

Urokinase activity

Urokinase activity in urine samples collected at different intervals was observed qualitatively in the above subjects.

PF-4

PF-4 activity was assayed in 10 subjects on blood samples collected before and after 3 h and 24 h of administration of piretanide and values were expressed in units per ml of plasma.

For the above parameters, the test subjects served as their own control.

Platelet aggregation

Nine subjects were studied for *in vitro* and *in vivo* experiments. For *in vitro* studies the final concentration of piretanide varied from 0·1–5 mM in which PRP was incubated at 37°C for 1 min prior to induction by ADP for aggregation. For *in vivo* studies, 6–12 mg piretanide was administered orally, blood was collected at intervals of 0 h to 1, 6, 12 and 24 h. A final concentration of 1×10^{-6} M ADP was used to induce platelet aggregation in all experiments. Platelet aggregation times (PAT) were recorded automatically along with the aggregation curves.

Statistical analysis of the results were obtained by using the Student's '*t*' test.

Results

Changes brought about in plasma fibrinolytic activity, fibrinogen and PF-4 by piretanide after 3 h of administration are depicted in the table 1. Following a single dose of 6 mg piretanide there was a significant rise in the mean euglobulin lytic activity, expressed as areas of lysis in mm^2, the peak being attained after 3 h ($P < 0.005$). The effect became evident within 1 h and persisted upto 24 h. The mean areas of lysis, from the 0 h to 1, 3, 6, 12 and 24 h were 222, 251, 312, 289, 267 and 249 mm^2, respectively. With rising fibrinolytic activity, plasma fibrinogen showed a mild fall which was not significant statistically. The mean plasma contents of fibrinogen, from 0 h to 1, 3, 6, 12 and 24 h were 291, 285, 256, 261, 265 and 289 mg/dl, respectively. Diuresis became manifest within 30–40 min and amounted to an average 300–500 ml. Urokinase activity in urine diminished within 30 min and it was gradually restored after $1\frac{1}{2}$–3 h. This effect of piretanide was similar to that of furosemide (Chohan *et al.*, 1977a).

Table 1. Changes in plasma fibrinolytic activity, fibrinogen and PF-4 3 h after a single oral dose of 6 mg piretanide.

Parameters	Initial		After piretanide		Significance
	Mean	SE	Mean	SE	
Fibrinolytic activity, area of lysis—mm² (13)	221·7	39·9	311·6	38·8	$P < 0.005$
Fibrinogen, mg/dl (9)	290·7	17·1	255·9	18·8	$P < 0.10$
Platelet factor—4, PF-4 units/ml (10)	0·164	0·028	0·118	0·014	$P < 0.001$

Numbers in parentheses indicate number of subjects.

PF-4 activity in plasma was significantly diminished 3 and 24 h after administration of piretanide compared to basal levels. PF-4 values, from 0 h to 3 and 24 h were 0·164, 0·118 ($P < 0.001$), and 0·148 ($P < 0.01$) units/ml plasma, respectively.

Primary platelet aggregation induced by ADP was progressively inhibited by prior addition of piretanide (f.c. ranged from 0·1–1·0 mM in the PRP) (figure 1A). However, 5 mM f.c. of the agent led to complete inhibition of platelet aggregation (figure 1B). When 5 mM piretanide was added just after platelet aggregation had been initiated by
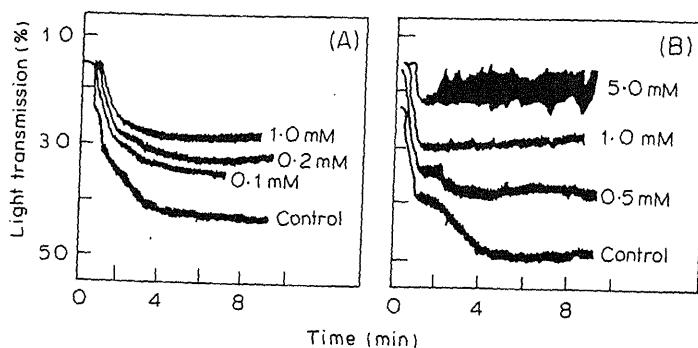
Figure 1. **A.** Inhibition of ADP-induced platelet aggregation by increasing concentrations of piretanide (0·1–1·0 mM) *in vitro.* **B.** Complete inhibition of ADP-induced platelet aggregation by 5 mM piretanide *in vitro.* The final concentration of ADP used is 10 μM.

ADP, disaggregation resulted immediately and secondary wave of aggregation did not occur (figure 2).
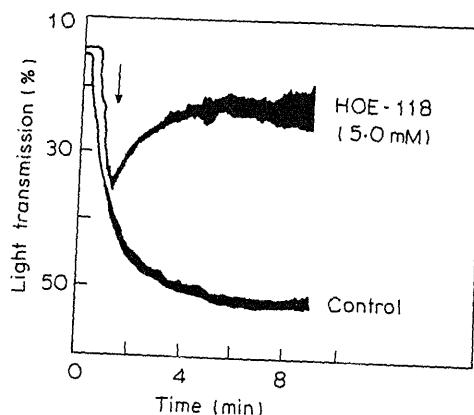
Figure 2. Reversal of ADP-induced platelet aggregation by piretanide (HOE-118, 5 mM) *in vitro.* The arrow indicates the moment of addtion of piretanide. The final concentration of ADP used is 10 μM.

After oral administration of 6 mg piretanide, ADP-induced platelet aggregation was inhibited generally within 1 h and this effect lasted beyond 12 h and in some subjects the effect persisted upto 24 h. Figure 3 shows a typical curve of inhibition of platelet aggregation in a subject after 1 h and 45 min of administration of 6 mg piretanide. Secondary wave of aggregation did not result. Platelet aggregation time was prolonged from 29 to 39 s in this experiment. Figure 4 depicts inhibition of platelet aggregation which persisted beyond 12 h after administration of 12 mg piretanide in this subject. The PAT prolonged from 20 to 25 s (left side curve). The inset on the right upper side in this figure shows an *in vitro* experiment in which PRP was obtained from the same subject prior to administration of the drug. It was incubated with 2·5 mM piretanide
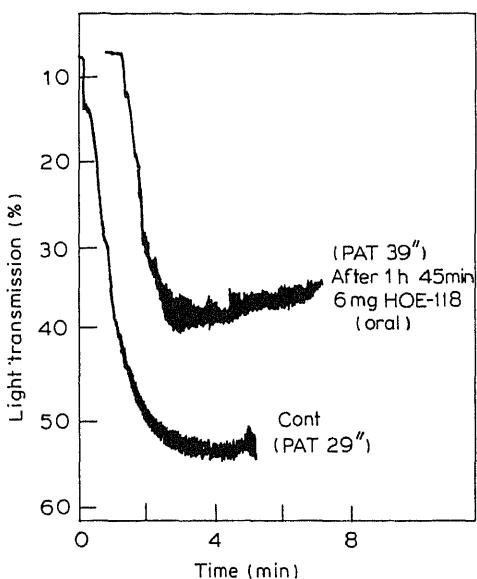
Figure 3. Inhibition of ADP-induced platelet aggregation following administration of a single oral dose of 6 mg piretanide (HOE-118). PAT prolonged from 29 s (control) to 39 s (test). The final concentration of ADP used is $10 \mu M$.
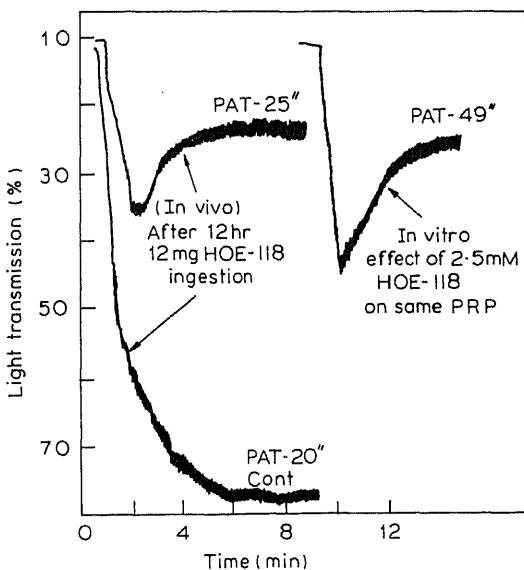
Figure 4. Left side curves: Persistence of inhibition of ADP-induced platelet aggregation beyond 12 h following administration of a single oral dose of 12 mg piretanide (HOE-118). Right upper side curve shows *in vitro* inhibition of platelet aggregation by 2·5 mM concentration of piretanide on the PRP obtained prior to ingestion of piretanide in the same subject. The f.c. of ADP used is $10 \mu M$ in both the situations.

(f.c.) and this led to an inhibition of ADP-induced platelet aggregation. This curve compared to the curve of inhibition obtained after ingestion of piretanide (left upper curve) shows that the *in vivo* inhibitory effects of 12 mg piretanide are more potent than that of 2·5 mM piretanide *in vitro*.

Discussion

The present study shows that piretanide, a potential diuretic, on oral administration in smaller doses of 6 mg, leads to a significant increase of plasma euglobulin fibrinolytic acitivity, a mild fall in fibrinogen, diminished urinary urokinase activity initially, and a decrease of PF-4 release from platelets in circulation. Piretanide also inhibits primary platelet aggregation both *in vivo* and *in vitro*. The rapid reversal of ADP-induced platelet aggregation by piretanide suggests induction of platelet relaxation. The platelet contractility, aggregation and release reaction are events of the same process and are governed by cAMP. cAMP inhibits the synthesis of prostaglandin endoperoxides (PGG_2) and thromboxane A_2, which induce aggregation and secretion of human platelets (Malmsten *et al.*, 1976). Furosemide, like several inhibitors of platelet aggregation, achieves these results by its cAMP-phosphodiesterase inhibitory and adenyl cyclase stimulatory properties (Chohan, 1980, 1984). A close parallelism between furosemide and piretanide would suggest a similar mechanism of action, though this remains to be elucidated for the latter.

The increase in plasma fibrinolytic activity by piretanide seems to have resulted from a spill over of a plasminogen activator or urokinase from renal parenchyma into general circulation.

Significant inhibition of platelet aggregation was achieved with 12 mg of piretanide. This effect persisted beyond 12 h and proved far better than that of 2·5 mM piretanide in *in vitro* experiment (figure 4). Assuming a plasma volume of 3,000 ml for an average person, this low dose would amount to a maximal concentration of 12 μM of piretanide in circulation. Since the *in vitro* concentration of the drug required to achieve similar degree of inhibition was 100 fold higher, it may be assumed that certain metabolic products of piretanide are involved in eliciting these inhibitory effects *in vivo*.

Activation of fibrinolysis and restriction of platelet activity and limiting release of PF-4 (possessing heparin-neutralising capacity) are of great importance to ensure patency of the microvasculature and prevent platelet thrombus formation. Defective fibrinolytic activity has been demonstrated in a number of vascular diseases, involving both veins and arteries, including the coronary vessels (Almer and Nilsson, 1975; Walker *et al.*, 1977) and invariably it is associated with increased platelet aggregation/adhesion, as observed in cardiopulmonary disorders, pulmonary oedema and hypertension of high altitude (Singh and Chohan, 1972a,b, 1973, 1974). An increased amount of PF-4 activity has been encountered after myocardial infarction (Holger-Madsen, 1960; Cotton *et al.*, 1968; Farbiszewski *et al.*, 1968; O'Brien *et al.*, 1975), in peripheral arteriosclerosis (Cotton *et al.*, 1972), in rheumatoid arthritis (Cotton and Johnson, 1968), in metastatic cancer (Gjesdal and Abrahamsen, 1976), and after surgery (Gjesdal and Sorlie, 1975; Godal and Fichera, 1961). In the above vascular disorders, therefore, a therapeutic approach of combining fibrinolytic and anti-platelet

agents, would be logical, to achieve dissolution of platelet microthrombi and facilitate better tissue perfusion.

A large number of compounds have been used to enhance fibrinolytic activity (von Kaulla, 1975), either with transient benefits or immunological side effects (Davidson and Walker, 1979). No synthetic agents have been reported to possess a combination of properties of activating the natural fibrinolytic system and inhibiting the platelet aggregation and PF-4 release at the same time, except furosemide (Chohan, 1980, 1984) and the presently studied drug piretanide (Chohan, 1982). Effectiveness with smaller dose and the ease of oral administration of piretanide are additional advantages. Apart from its usefulness as a potential diuretic, piretanide would be worthy of trial in conditions where fibrinolytic activity is reduced and platelet aggregation and PF-4 are increased.

Acknowledgement

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Prevention of aortic lesions and hyperlipidaemia by alfalfa seed extract in cholesterol fed rabbit

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Abstract. Extract of alfalfa seed (ethanolic 50% v/v) prevents the development of plaque formation and hyperlipidaemia in cholesterol fed rabbits. It inhibits the elevation of serum total cholesterol, triglycerides, phospholipids, LDL-cholesterol and total cholesterol/phospholipid ratio, while HDL-cholesterol/total cholesterol ratio increases, which is associated with a reduced incidence of atherosclerosis. Further reduction in total cholesterol and phospholipid contents of liver and heart muscle are suggestive of a beneficial role of the seed extract. The possible mechanisms of action are discussed.

Keywords. Alfalfa seed extract (ethanolic 50% v/v); HDL-cholesterol; atherosclerosis; hyperlipidaemia; LDL-cholesterol.

Introduction

The relationship between lipid and atherosclerosis has been a subject of extensive investigation over the last 6 decades. Cholesterol added to diet (0·5–2%) results in hypercholesterolemia (Shore *et al.*, 1974; Wissler and Verselinovitch, 1974) and subsequently in the induction and development of atherosclerosis (Brown *et al.*, 1965).

Alfalfa meals prevent hypercholesterolemia in rabbits (Yanaura and Sakamoto, 1975) and cynomologus monkeys (Malinow *et al.*, 1978). Antiatherosclerotic activity of alfalfa seed extract (ethanolic 50% v/v) in chicks has been reported from our laboratory (Dixit and Joshi, 1985).

The aim of the present study was to examine whether alfalfa seed extract feeding prevents of aortic lesion and hypercholesterolemia in cholesterol fed rabbits.

Materials and methods

Medicago sativa (alfalfa) seeds obtained from the Seed Corporation of India were powdered and defatted with petroleum ether (60–80°). Defatted material was subjected to Soxhlet extraction with Ethanol (50% v/v) for 24 h. Ethanol was removed under reduced pressure to obtain a brown solid.

Eighteen adult healthy rabbits were used. They were maintained in an airconditioned room (26° ± 1°C) and were divided into groups of 6 each. Group A served as controls. Group B received 500 mg cholesterol in 5 ml coconut oil/day for a period of 4 months.

Group C received 500 mg cholesterol in 5 ml coconut oil/day + 500 mg of ethanolic (50%) extract of alfalfa seeds/day.

All animals were killed after a 4 month period. The blood was collected through cardiac puncture, serum separated and stored at − 20°C until assayed. Total cholesterol (Zlatkis *et al.*, 1953), triglyceride (Gottfried and Rosenberg, 1973), phospholipid (Zilversmit and Davis, 1950), HDL-cholesterol (Burnstein *et al.*, 1976) and LDL-cholesterol (Dedonder-Decoopman *et al.*, 1980) were estimated and statistically analysed using Student's '*t*' test.

Aorta, liver and heart muscles were quickly removed, cleared of fat and connective tissue. Aortas were prepared for histopathological examinations. The liver and left ventricular heart muscles were frozen and analysed for glycogen (Montgommery, 1957), cholesterol (Zlatkis *et al.*, 1953), phospholipid (Zilversmit and Davis, 1950) and triglyceride (Gottfried and Rosenberg, 1973).

Toxicity Studies

Male rats of inbred colony (body wt. 170 ± 13 g) were assigned randomly to 3 groups of 8 animals each. These groups were maintained on rat feed pellets (Hindustan Lever Ltd.). After first 4 days, 1% cholesterol was added to the diets of group 2 and 3 animals. Group 1 served as control, while groups 2 and 3 were administered orally 100 mg and 300 mg/day of the ethanolic extract of alfalfa seed for 2–6 months. All animals of group 3 and half each from groups 1 and 2 were killed after 2 months, while the remaining were killed after 6 months.

No deaths occurred during the experiments and seed extract had no inhibitory effect on body weight. Serum cholesterol and triglycerides were found to be low. At post-mortem, no gross pathological changes were observed.

Results

Biochemical changes

The body and liver weights were reduced significantly in rabbits fed with cholesterol + alfalfa simultaneously (Group C) when compared with Group B (table 1). The results presented in table 2 indicate that the cholesterol contents of liver and heart muscles of rabbits fed with cholesterol + alfalfa seed extract were significantly reduced. No significant change in triglyceride contents of liver was noticed, but a significant reduction was noticed in that of the heart muscle. Phospholipid contents of liver and heart muscles were reduced significantly. Glycogen contents of liver and heart muscles were also reduced. A significant reduction in serum total cholesterol, phospholipid, triglyceride, LDL-cholesterol, VLDL-cholesterol and total cholesterol/phospholipid ratio was observed in rabbits fed with cholesterol + alfalfa (Group C) compared to cholesterol feeding alone (Group B), while HDL-cholesterol/total cholesterol ratio was raised after combined feeding (table 3).

Histopathological changes in aorta

Cholesterol fed rabbits showed lesions in aortic arch characterised by a thickened intima, cell proliferation, collagen and abundant lipid accumulation (figure 1).

Table 1. Changes in the body, liver and heart weight after cholesterol/alfalfa seed extract feeding.

Treatment	Body weight (kg)		Liver	Heart
	Initial	Final	(mg/100 gm body weight)	
Group A (control)	1·68 ± 0·09	1·62 ± 0.08	2603 ± 26.15	203·0 ± 7·0
Group B (cholesterol feeding)	1·66 ± 0·1	1·47 ± 0·06	4216·0 ± 64·0	213·60 ± 4·37
Group C (cholesterol + alfalfa feeding)	1·43 ± 0·1	1·08 ± 0·11[c]	2661·9 ± 40·2[b]	198·69 ± 7·6[a]

[a] *P*: Not significant when Group C is compared with Group B.
[b] *P* ≤ 0·01 when Group C is compared with Group B.
[c] *P* ≤ 0·001 when Group C is compared with Group B.
The values represent Mean ± SEM.

Calcification of tunica media was conspicuous. Such alterations did not occur in the aorta of rabbits which were maintained on simultaneous feeding with cholesterol and alfalfa seed extract (figure 2).
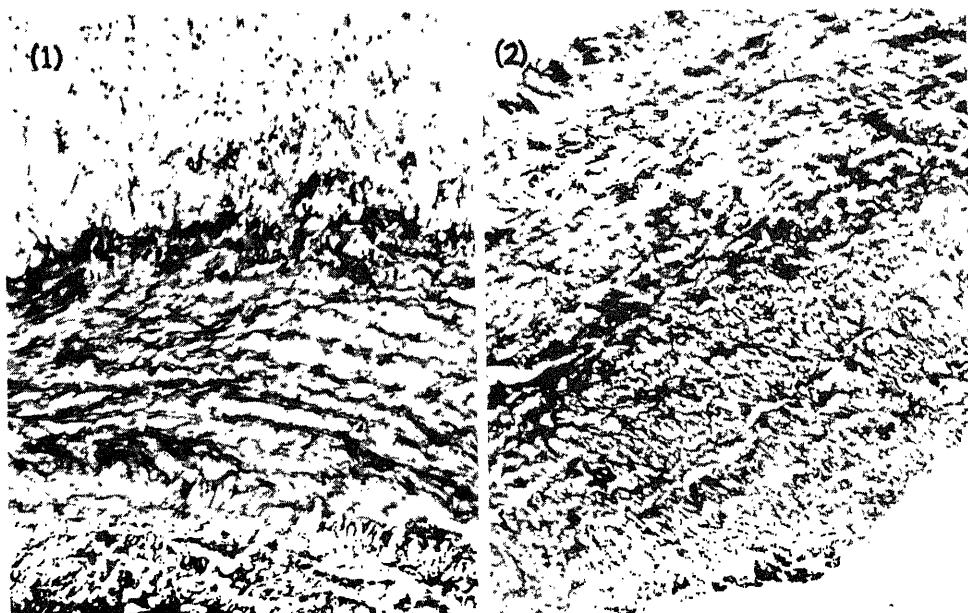
Figures 1 and 2. **1.** Aorta of cholesterol fed rabbit showing cell proliferation, collagen and lipid accumulation. × 100 HE. **2.** Aorta of rabbit fed with cholesterol and alfalfa seed extract. Note the normal architecture of the aortic wall. × 100 HE.

Table 2. Changes in tissue lipids after cholesterol/alfalfa seed extract feeding.

Treatment	Cholesterol (mg/gm)		Triglyceride (mg/gm)		Phospholipid (mg/gm)		Glycogen (mg/gm)	
	Liver	Heart muscle	Liver	Heart muscle	Liver	Heart muscle	Liver	Heart muscle
Group A (control)	10·93±0·15	9·12±0·07	3·96±0·05	4·37±0·18	8·32±0·08	10·02±0.08	5·71±0·04	1·10±0·09
Group B (cholesterol feeding)	15·5±0·3	17·25±0·25	5·50±0·20	6·0±0·2	13·0±0·10	11·5±0·20	5·73±0·14	2·85±0·15
Group C (cholesterol + alfalfa feeding)	11·34±0·06^c	9·5±0·03^c	5·34±0·06^a	5·02±0·03^b	9·25±0·07^c	10·71±0·06^b	5·09±0·005^b	1·23±0·007^c

^{a} P: Not significant when Group C is compared with Group B.
^{b} $P \leqslant 0.01$.
^{c} $P \leqslant 0.001$.
The values represent Mean ± SEM.

Table 3. Serum analysis of cholesterol/alfalfa seed extract fed rabbits.

Treatment	Total cholesterol (mg/dl)	HDL-cholesterol (mg/dl)	Triglyceride (mg/dl)	VLDL-cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	Phospholipid (mg/dl)	HDL-cholesterol/total cholesterol ratio	Total cholesterol/phospholipid ratio
Group A (control)	114·6±8·8	49·66±3·28	58·18±4·36	12·31±0·99	61·58±3·6	126·6±3·42	0·433±0·04	0·903±0·25
Group B (cholesterol feeding)	768±8·5	122·88±0·255	179·1±4·16	35·83±1·68	609·17±10·16	267·6±5·32	0·16±0·03	2·869±0·15
Group C (cholesterol + alfalfa feeding)	156±29·0[a]	74·88±1·682[a]	76·33±24·36[a]	15·26±4·87[a]	71·73±41·65[a]	135·1±20·06[a]	0·48±0·058[a]	1·154±0·145[a]

[a] $P \leqslant 0.001$ when Group C is compared with Group B.
The values represent Mean ± SEM.

Discussion

The ethanolic extract of *Medicago sativa* seeds contain two main aglycones namely, medicagenic acid and hederagenin (Malinow, 1984). Alfalfa seed extract prevents the development of plaque formation and hyperlipidaemia. The possible mechanism for prevention of plaque formation may be that HDL-inhibits the LDL-arterial wall uptake and also facilitate the transport of cholesterol from peripheral tissue to the liver, where it is catabolised and excreted out of the body (Carew *et al.*, 1976).

Reduction in hyperlipidaemia occurs simultaneously with an increase in the HDL-cholesterol/total cholesterol ratio which is associated with a reduced incidence of atherosclerosis (Castelli *et al.*, 1977). Decrease in total cholesterol-phospholipid ratio after alfalfa feeding indicates the antiatherogenic nature of the plant product. Further reduction in total cholesterol and phospholipid contents of liver and heart muscle may be suggestive of a beneficial role for the drug.

Malinow *et al.* (1980a, b) suggested that alfalfa ingestion decreased the intestinal absorption and exogenous and endogenous cholesterol and increased the bile acid excretion. Jackson (1981) demonstrated that alfalfa meal contain high levels of an immunoreactive thyrotropin releasing hormone like material (IR-TRH), a finding that suggests another possible mechanism of action.

Finally it seems that the ethanolic extract (50% v/v) of alfalfa seed is beneficial in reducing hyperlipidaemia and prevents atherosclerosis. The apparent lack of toxicity advocates its long term use. However, long term tolerance studies are needed before being recommended for human use.

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Purification and characterization of proteolytic enzymes from normal and opaque-2 *Zea mays* L. developing endosperms*

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Abstract. Purification and characterization of proteases from developing normal maize endosperm and high lysine opaque-2 maize endosperm have been carried out with a view to understand their role in storage protein modification. At day 15, normal maize endosperm had two types of proteolytic enzymes, namely, protease I and protease II, while at day 25 protease II disappeared and in place protease III appeared. However, in opaque-2 maize endosperm at both the stages only one type of enzyme (protease I) was present. These proteases had many properties in common—optimum pH and temperature were respectively, 5·7 and 40°C; their activity was inhibited to the extent of 75–93 % by *p*-chloromercuribenzoate; trypsin inhibitor inhibited the activity more at early stages of endosperm development; all proteases cleaved synthetic substrates *p*-tosyl-L-arginine methylester and N-benzoyl-L-tyrosine ethyl ester and poly-L-glutamic acid. The K_m values of day 15 and 25 normal maize endosperm proteases ranged from 2·73–3·30, while for opaque-2 maize endosperm protease I it was 3·33 mg azocasein per ml assay medium. These enzymes, however, differed with respect to proteolytic activity towards poly-L-lysine. Only normal maize endosperm protease III at day 25 followed by protease II at day 15 showed high activity towards this homopolypeptide suggesting thereby their role in determining the quality of normal maize endosperm protein.

Keywords. *Zea Mays*; Gramineae; proteases; esterolytic activity; homopolypeptides.

Introduction

Maize endosperm protein is considered to be nutritionally poor because of its deficiency in lysine and tryptophan. In the presence of opaque-2 gene, nutritional quality of maize protein is improved mainly because of suppressed synthesis of zein (Murphy and Dalby, 1971; Lodha *et al.*, 1978), which is extremely deficient in lysine and tryptophan. However, as the grain matures, nutritional quality of not only normal maize protein but also of opaque-2 maize deteriorates (Gupta *et al.*, 1978). Decrease in lysine per endosperm towards maturity has been reported in normal and opaque-2 maize (Gupta *et al.*, 1977; Lodha *et al.*, 1978), which may be due to protein turnover or selective degradation of lysine-rich proteins. Sodek and Wilson (1970) reported conversion of injected lysine to glutamic acid and proline more in normal maize endosperms than in opaque-2 maize endosperms during development. In our earlier

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study (Ram *et al.*, 1986) based on degradation of [^{3}H]-lysine and [^{14}C]-leucine labelled protein *in vitro* by proteases from developing normal and opaque-2 maize endosperms, it was suggested that lysine-specific proteases may be present in both the normal and opaque-2 developing maize endosperms. In the present investigation, purification and characterization of proteolytic enzymes from developing normal and opaque-2 maize endosperms have been carried out.

Materials and methods

A normal maize inbred line Fla 3H94 and its opaque-2 isogenic line were obtained from the All India Coordinated Maize Improvement Project, IARI, New Delhi. Self-pollinated ears were harvested at 15, 20, 25 and 30 days after pollination and endosperms were collected after removing the pericarp and embryo from the kernels at 0–4°C. The endosperms were stored in deep freeze till further use.

Extraction of proteases

Endosperms were hand ground in liquid nitrogen in a pestle and mortar using sterilized sand with medium (1:2, W/V) containing 50 mM potassium phosphate buffer (pH 7·5), 35 mM EDTA, 10 mM dithiothreitol (DTT). The homogenate was centrifuged for 15 min at 20,000 *g* and the supernatant was used as crude enzyme. All the operations unless otherwise stated were carried out at 4°C.

Assay of acid protease activity

Acid protease activity was assayed according to Miller and Huffaker (1981) with slight modifications. The reaction mixture in a final volume of 2·2 ml contained: 50 mM potassium phosphate buffer (pH 5·7), 1·0 ml, azocasein (4 mg/ml of 50 mM phosphate buffer, pH 5·7), 1·0 ml, and enzyme preparation, 0·1–0·2 ml. After incubation at 40°C for 1 h, 1·0 ml of 14 % perchloric acid (PCA) was added to stop the reaction. Tubes were kept for 1 h at 0°C for ageing and then centrifuged. Hydrolysis was measured as an increase in absorbance of supernatant at 340 nm. In the blank 1·0 ml 14 % PCA was added before adding the enzyme.

Enzyme unit

One unit of activity was defined as the amount of enzyme which caused an absorbance change of 0·01/h measured at 340 nm. The specific activity of enzyme was expressed as enzyme units per mg protein.

Purification of proteases

Ammonium sulphate fractionation: Solid ammonium sulphate was added slowly to the crude extract with constant stirring till 70 % saturation was obtained. The extract was allowed to stand for 1 h in deep freeze. The pellet recovered by centrifugation at 20,000 *g* for 15 min was resuspended in extraction buffer. It was dialysed for 16 h against the extraction buffer at 4°C. Two changes of the buffer were made. The recovery of protease enzyme was 80 %.

DEAE–cellulose chromatography: Dialysed supernatant (18–20 ml) from the above step was layered on DEAE–cellulose column (2·5 × 15 cm) equilibrated with 0·05 M potassium phosphate buffer (pH 7·5). After washing the sample with 2 ml of buffer, elution was first done with 110 ml of 0·06 M phosphate buffer (pH 7·5) and then with 120 ml of 0·07 M phosphate buffer (pH 7·5). The effluent was monitored at 280 nm using LKB uvicord-II and 2·2 ml fractions were collected. Flow rate was maintained at 30 ml per h. Each fraction was assayed for protease activity.

Characterization of purified proteases

Two forms of proteases from normal endosperm and one from opaque-2 endosperm at 15 and 25 day stages obtained as above were characterized. For determining the optimum pH, temperature, incubation time and substrate concentration, assay mixture and other conditions of the assay were the same as used for the assay of crude enzyme.

Hydrolysis of homopolypeptides by proteases

The hydrolysis of homopolypeptides such as poly-L-lysine [molecular weight (M_r) 6000] and poly-L-glutamic acid $(M_r$ 10,000), was studied using an assay mixture which contained: purified enzyme, 0·1 ml, 50 mM phosphate buffer (pH 5·7) containing 1 mM DTT, 0·4 ml; and homopolypeptide solution (1 mg homopolypeptide/ml phosphate buffer), 0·5 ml. The reaction mixture was incubated at 40°C for 1 h. The reaction was stopped by adding 1 ml of 14% PCA. In control, 1 ml 14% PCA was added at zero time. After centrifugation, 1 ml of supernatant solution was layered on Sephadex G-50 column (1 × 30 cm) equilibrated with phosphate buffer (pH 7·5). The elution buffer used was 50 mM phosphate buffer (pH 7·5) and the flow rate was 10 ml per h. Two ml fractions were collected. The concentration of proteolysed products in the eluted fractions was estimated by ninhydrin reaction as described by Moore and Stein (1954).

Esterolytic activity

Esterolytic activity of the purified enzymes towards synthetic substrates such as *p*-tosyl-L-arginine methyl ester (TAME) and N-benzoyl-L-tyrosine ethyl ester (BTEE) was determined as follows: TAME (1 mM, 1·0 ml) was added to a mixture of 0·1 ml of purified enzyme solution and 0·9 ml of 50 mM phosphate buffer (pH 5·7) containing 1 mM DTT. The cleavage was determined from the change in absorbance at 247 nm against a control. Similarly, cleavage of BTEE was determined from the change in absorbance at 256 nm.

Effect of inhibitors

In order to study the effect of PCMB, 0·1 ml purified enzyme preparation was pre-incubated with 1·0 ml of 0·063 M *p*-chloromercuribenzoate (PCMB) in 50 mM potassium phosphate buffer (pH 5·7) for 15 min at 40°C. Then it was incubated with 1 ml azocasein (4 mg/ml) solution and protease activity with and without PCMB, was determined as described earlier.

In the case of trypsin inhibitor study, the assay mixture in a final volume of 2 ml

contained: 1 ml azocasein solution, 1 ml inhibitor solution (Sigma Type II-S soybean trypsin inhibitor, 250 μg/ml of 50 mM potassium phosphate buffer, pH 5·7) and 0·1 ml enzyme preparation. Protease activity with and without the inhibitor, was determined as described earlier.

The results presented are an average of at least two experiments, each having been carried out in triplicate.

Results

Protease activity

Acid protease activity per endosperm with azocasein as substrate remained constant till day 20, increased at day 25 and decreased thereafter at day 30 in normal maize, while in opaque-2 endosperms it increased linearly upto day 25 and then declined at day 30. The activity was higher at days 15 and 25 and lower at days 20 and 30 in normal maize than in opaque-2 maize endosperm (figure 1).
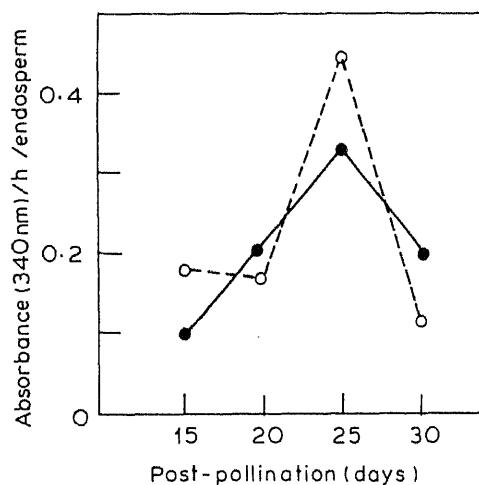
Figure 1. Acid protease activity with azocasein in the developing endosperm of normal (O) and opaque-2 (●) maize.

Purification of proteases

Proteases from normal and opaque-2 endosperms of days 15 and 25 post-pollination stages were purified using ammonium sulphate fractionation and DEAE-cellulose column chromatography. The results are presented in table 1 and figure 2.

Twenty six to eighty five fold purification was obtained for different proteases. Three forms of proteases, namely, protease I, II and III were isolated. Protease III (figure 2C) was present only at day 25 and protease II (figure 2A) at day 15 in normal maize. Opaque-2 endosperm at both the stages had only one major peak of protease I

Table 1. Purification of proteases.

Preparation	Total activity (units)	Total protein (mg)	Specific activity	Purification (fold)
Day 15 normal maize endosperm				
Crude extract	3120	190	16	0
Ammonium sulphate fraction	2800	82	34	2
Pooled DEAE fractions (42–46)—Protease II	1100	2·6	423	26
Pooled DEAE fractions (84–88)—Protease I	1615	1·3	1242	78
Day 15 opaque-2 endosperm				
Crude extract	2720	178	15	0
Ammonium sulphate fraction	2070	57·2	36	2·4
Pooled DEAE fractions (81–85)—Protease I	1540	1·2	1283	85
Day 25 normal maize endosperm				
Crude extract	3510	184	19	0
Ammonium sulphate fraction	2755	60·8	45	2·4
Pooled DEAE fractions (12–16)—Protease III	1870	1·3	1438	76
Pooled DEAE fractions (71–75)—Protease I	·770	1·4	550	29
Day 25 opaque-2 endosperm				
Crude extract	2613	171·6	15	0
Ammonium sulphate fraction	2070	55·8	37	2·5
Pooled DEAE fractions (87–91)—Protease I	1540	1·3	1184	79

Enzyme unit: One unit of enzyme activity caused an absorbance change of 0·01/h at 340 nm.

(figure 2B, D). Protease I was also present at both the stages in normal maize endosperms. Protease II and III were eluted with 0·06 M phosphate buffer (pH 7·5), while protease I with 0·07 M phosphate buffer (pH 7·5).

Characterization of proteases

The purified fractions of proteases obtained from DEAE-cellulose column were characterized with respect to optimum pH, temperature, incubation time, substrate concentration, esterolytic activity, hydrolysis of homopolypeptides, stability and the effect of inhibitors. The results are presented in tables 2 and 3 and in figures 3 and 4.

The activity of proteases from both normal and opaque-2 endosperms was linear upto 90 min with an optimum of pH 5·7 and optimum temperature of 40°C. Therefore, in all subsequent studies, an incubation time of 60 min was used and assay done at the optimum pH and temperature.
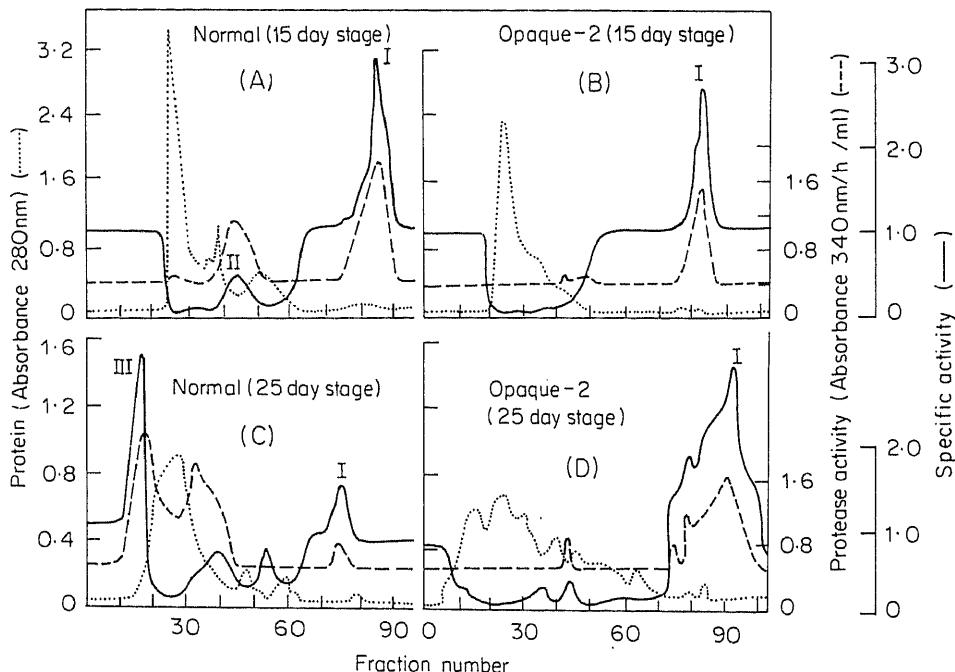
Figure 2. Elution profile of normal and opaque-2 maize endosperm proteases from DEAE-cellulose column.

Table 2. Activity of normal and opaque-2 maize endosperm proteases on synthetic substrates.

| | Activity (Δabsorbance/h/ml) | |
Enzyme	TAME	BTEE
	15 day stage	
Normal maize protease II	0·60	0·55
Normal maize protease I	0·60	0·60
Opaque-2 protease I	0·63	0·70
	25 day stage	
Normal maize protease III	0·45	0·50
Normal maize protease I	0·50	0·46
Opaque-2 protease I	0·45	0·66

Substrate concentration and K_m: The effect of substrate (azocasein) concentration on the velocity of the reaction was characterized by a hyperbolic curve. The K_m for each protease was calculated from the Lineweaver–Burk plot. The K_m values for 15 day stage normal protease II, normal protease I and opaque-2 protease I were 3·30, 2·82 and

Table 3. Effect of different storage temperatures on the activity of normal and opaque-2 maize endosperm proteases.

Enzyme	Initial protease activity (Δ absorbance/h/ml)	Protease activity after 10 day storage (Δ absorbance/h/ml)		
		4°C	−20°C	−196°C
15 day stage				
Normal maize protease II	1·0	0·70	0·90	0·90
Normal maize protease I	1·55	1·0	1·30	1·28
Opaque-2 protease I	1·40	0·90	1·18	1·15
25 day stage				
Normal maize protease III	1·60	1·05	1·35	1·30
Normal maize protease I	0·80	0·65	0·75	0·73
Opaque-2 protease I	1·40	0·85	1·20	1·15

3·33 mg azocasein per ml, respectively. The K_m values for 25 day stage normal protease III, normal protease I and opaque-2 protease I were 3·0, 2·73 and 3·33 mg azocasein per ml, respectively.

Esterolytic activity: The esterolytic activities of all proteases, except day 25 opaque-2 protease I, with TAME and BTEE as substrate were similar. Day 25 opaque-2 protease I, however, preferred BTEE over TAME as substrate (table 2).

Specificity: The substrate specificity of normal and opaque-2 purified proteases was investigated using poly-L-lysine and poly-L-glutamic acid as substrates and the results are illustrated in figures 3 and 4.

All proteases cleaved poly-L-glutamic acid (figures 3 and 4). However, poly-L-lysine was a good substrate only for normal protease III from day 25 (figure 4C) and normal protease II from day 15 (figure 3C) endosperms. Day 15 normal protease I (figure 3A) and day 25 opaque-2 protease I (figure 4E) hydrolysed poly-L-lysine at a slower rate. No activity was observed with 25 day normal protease I (figure 4A) and 15 day opaque-2 protease I (figure 3E).

Enzyme stability: The enzymes were more stable when stored at −20°C and −196°C than at 4°C (table 3). Normal maize protease II at 15 day stage and protease I at 25 day stage were relatively more stable.

Inhibitors: PCMB at 0·03 M concentration inhibited all proteases to the extent of 75–93%. Trypsin inhibitor also inhibited the activity of normal and opaque-2 maize proteases. At the trypsin inhibitor concentration of 250 μg per 2·1 ml assay medium, the activities of day 15 normal maize protease I and II were inhibited to the extent of 70% as against 46% inhibition of 25 day stage normal maize protease I and III. At the same concentration of trypsin inhibitor the activity of opaque-2 protease I of 15 and 25 day stages was inhibited by 64% and 34%, respectively.
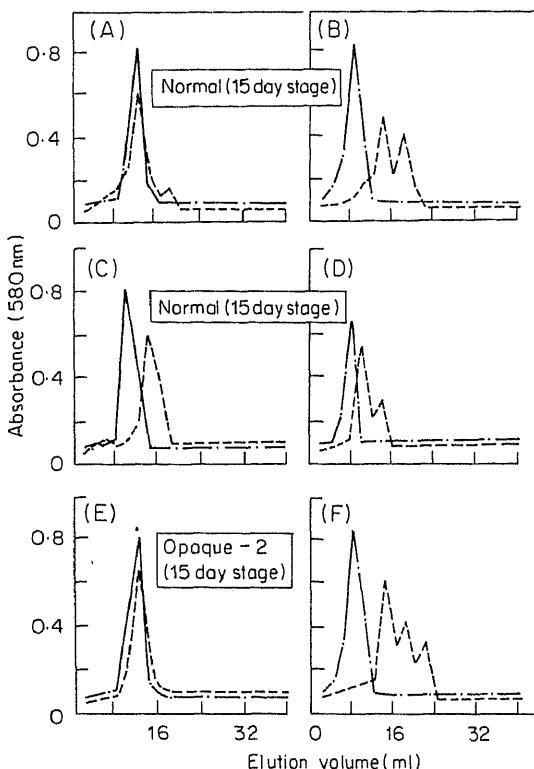
Figure 3. Action of proteolytic enzymes from 15-day old normal and opaque-2 maize endosperms on homopolypeptides. After the reaction was terminated, the supernatant solution was layered on Sephadex G-50 column. The presence of the polypeptides or their digestion products in the eluted fraction was determined by absorbance at 580 nm after reaction with ninhydrin. The cleavage of the homopolypeptides was ascertained by the disappearance or reduction in their size. (———), Homopolypeptides treated with inactive enzyme and (——————), with active enzyme. A. Normal maize protease I on poly-L-lysine. B. Poly-L-glutamic acid. C. Normal maize protease II on poly-L-lysine. D. Poly-L-glutamic acid. E. Opaque-2 maize protease I on poly-L-lysine. F. Poly-L-glutamic acid.

Discussion

Proteases are known to play an important role in the breakdown of proteins and in protein turnover in germinating cereal seeds. The acid protease from maize endosperm has been shown to be active against native proteins zein and glutelin (Harvey and Oaks, 1974). Our earlier study has suggested the presence of lysine-specific proteases in both normal and opaque-2 developing endosperms (Ram *et al.*, 1986). The present study showed the presence of two proteases, protease I and II in 15-day old normal maize endosperm, and appearance of protease III in place of protease II at 25 day stage. In case of opaque-2 maize endosperm only one type of protease (protease I) has been found to be present at both 15 and 25-day stages. All the proteases cleaved poly-L-
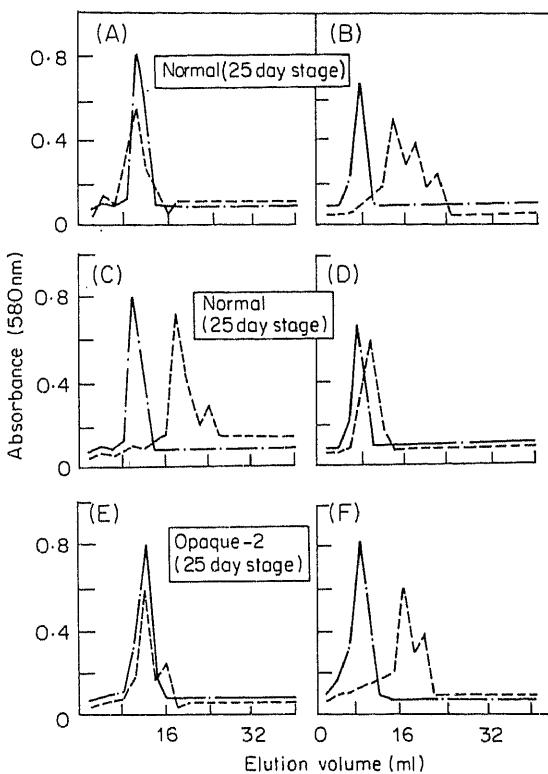
Figure 4. Action of proteolytic enzymes from 25-day old normal and opaque-2 maize endosperms on homopolypeptides. Experimental details are same as in figure 3. **A.** Normal maize protease I on poly-L-lysine. **B.** Poly-L-glutamic acid. **C.** Normal maize protease III on poly-L-lysine. **D.** Poly-L-glutamic acid. **E.** Opaque-2 protease I on poly-L-lysine. **F.** Poly-L-glutamic acid.

glutamic acid. As regards proteolytic specificity towards poly-L-lysine, only 25-day normal protease III followed by 15-day normal protease II showed high activity, while 15 day normal maize protease I and 25 day opaque-2 protease I showed only a minor activity. Esterolytic activity with synthetic substrates TAME and BTEE suggested that all the proteases had this activity like trypsin and chymotrypsin.

The biological value of normal maize proteins deteriorates more as compared to opaque-2 maize protein during the later stages of grain maturation (Gupta *et al.*, 1978). This is mainly due to the increased synthesis of lysine-poor proteins during later stages of endosperm development (Murphy and Dalby, 1971; Lodha *et al.*, 1978), protein turnover and/or removal of lysine from performed proteins and its conversion to other amino acids (Sodek and Wilson, 1970). Synthesis of protease III with high activity for poly-L-lysine in the later stages of normal maize endosperm development would favour degradation of lysine-rich proteins or will result in post-translational modifications where lysine-rich residues are removed. This is consistent with the observation that during this period inspite of substantial accumulation of storage protein in endosperm,

the absolute amount of lysine per endosperm decreases (Gupta *et al.*, 1977; Lodha *et al.*, 1978). This has also been confirmed by rat feeding trials, wherein the protein quality of normal maize kernals at day 25 has been shown to be equal to that of opaque-2 maize at maturity (Gupta *et al.*, 1978). The absence of protease III in opaque-2 maize endosperm would favour lack of such modifications as may perhaps occur in normal maize endosperm. Ghosh and Das (1980) have also reported that in wheat, protease C, which appears late in endosperm development, cleaves poly-L-lysine. Therefore, the results of this study along with the results obtained in an earlier study (Ram *et al.*, 1986) are suggestive of the role of proteases in protein turnover/post-translational modifications of storage proteins in normal maize endosperm.

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Ammonium ions prevent methylation of uridine to ribothymidine in *Azotobacter vinelandii* tRNA

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Abstract. It was shown that tRNA from *Azotobacter vinelandii* grown in the presence of ammonium chloride lacks ribothymidine while that grown in the absence of the ammonium salt contains this modified nucleoside. [^{32}P]-Labelled tRNA from this organism grown in a medium containing the ammonium salt was digested with RNase T_1 and the pseudouridine-containing tetranucleotide, common to all tRNAs was isolated and analysed for the nucleoside replacing the ribothymidine. It was found to be uridine. Cells previously labelled with [^{32}P]-phosphate in the ammonium salt medium were washed and incubated in the ammonium salt-free medium to test whether ribothymidine would be formed upon removal of the ammonium ions. Methylation of the uridine did not take place.

Keywords. Ribothymidine; uridine methylation; tRNA modification; *Azotobacter vinelandii* tRNA.

Introduction

The metabolism of some of the modified nucleosides in the tRNA of microorganisms is known to be sensitive to culture conditions. Species whose relative proportions change with the growth conditions include 2-methylthio-N^6-isopentenyl-adenosine (Buck and Griffiths, 1981; Buu *et al.*, 1981), 2-methylthioribosylzeatin (Ajitkumar and Cherayil, 1985a; Buck and Ames, 1984; Morries *et al.*, 1981), 5-methylaminomethyl-2-thiouridine (Chackalaparampil and Cherayil, 1981) and 4-thiouridine (Ajitkumar and Cherayil, 1985b). We have observed that when the nitrogen fixing bacteria, *Azotobacter vinelandii* and *Rizobium meliloti*, are grown in the presence of an ammonium salt the tRNA from these organisms lacks ribothymidine (T). On the other hand, when *A. vinelandii* is grown in the absence of an ammonium salt its tRNA contains T to the extent of 1 mol/mol (Ajitkumar and Cherayil, 1982). T, which occurs at the TψC arm of tRNAs is one of the unique modified nucleosides present in most tRNAs, except the initiator methionine tRNA from many eukaryotes (Gauss and Sprinzl, 1983). There are systems in which the tRNA naturally lacks T. Such tRNAs have been shown to be as good as those which contain T in *in vitro* protein synthesis (Svensson *et al.*, 1971). However, it appears that T

Abbreviations used: T, Ribothymidine; ψ, pseudouridine; 2-DTLC, two dimensional thin-layer chromatography.

plays a subtle role in protein synthesis, although its exact function is not known (Kersten *et al.*, 1981). In certain systems T has been found to be replaced with 2'-O-methylribothymidine or 2-thioribothymidine (Gauss and Sprinzl, 1983). The content of the latter has been reported to increase with temperature in certain thermophilic organisms (Watanabe *et al.*, 1980).

The present study was carried out with the main objective of identifying the nucleoside which replaces T when *A. vinelandii* is cultured in a medium containing an ammonium salt.

Materials and methods

Carrier-free $H_3{}^{32}PO_4$ was obtained from Bhabha Atomic Research Centre, Bombay. RNases T_1 and T_2 were from Sigma Chemical Co., St. Louis, Missouri, USA. Plastic backed thin layer microcrystalline cellulose plates and DEAE-cellulose paper were purchased from Macherey-Nagel, and Schleicher and Schull, Federal Republic of Germany, respectively.

Growth of bacteria and isolation of tRNA

A. vinelandii OP (Wisconsin) was grown in the absence and in the presence of an ammonium salt. In the first case Burk-modified nitrogen-free medium (Strandberg and Wilson, 1967) was used and in the second case 2·8 mg N/ml or 0·49 mg N/ml as NH_4Cl was added to the nitrogen-free medium. Concentrations of the ammonium salt used were sufficient to completely repress the nitrogenase gene (Gordon and Brill, 1972). Cells were grown in low phosphate medium in the presence of $[^{32}P]$-phosphate and the labelled tRNA was isolated and purified as described previously (Ajitkumar and Cherayil, 1982).

Analysis of $[^{32}P]$-labelled products

$[^{32}P]$-Labelled tRNA was converted to nucleotides by digestion with RNase T_2 and to oligonucleotides by digestion with RNase T_1. Two dimensional thin-layer chromatography (2-D TLC) of nucleotides was carried out on 10 × 10 cm cellulose plates, 0·1 mm thickness, using the solvent systems isobutyric acid:0·5 M ammonium hydroxide, 5:3 (v/v) in the first dimension and isopropanol:conc. HCl:water, 70:15:15 (v/v/v) in the second dimension (Nishimura *et al.*, 1967). The plates were dried and subjected to autoradiography. Separation of 3'-phosphates of T, uridine and pseudouridine (ψ) was effected by descending paper chromatography on Whatman 1 mm paper using isopropanol:water:conc. ammonia, 70:30:1 (v/v/v) as the solvent system (Brownlee, 1972). High voltage electrophoresis was carried out on Whatman 3 mm paper or DEAE-cellulose paper in acetic acid-pyridine-EDTA buffer, pH 3·5, at 40–60 V/cm using xylene cyanol and acid fuchsin as dye markers (Smith, 1967). Radioactive samples were recovered from the Whatman paper and the DEAE-cellulose paper by elution with water and 1·0 M triethylammonium acetate, pH 10, respectively (Sanger and Brownlee, 1967).

Results

Effect of ammonium salt on ribothymidine production

In the earlier study, it was found that in *A. vinelandii* cells grown with excess of ammonium chloride (2·8 mg N/ml), the tRNA completely lacked T, while the cells grown in the absence of ammonium chloride contained T to the extent of 1 mol/mol of tRNA (Ajitkumar and Cherayil, 1982). In order to find out whether this was due to any effect of the excess of ammonium ions, the cells were grown with much less ammonium salt (0·49 mg N/ml), only slightly higher than that required to repress the nitrogenease gene (Gordon and Brill, 1972). RNase T_2 digest of $[^{32}P]$-labelled tRNA isolated from cells grown with low ammonium salt was subjected to 2-D TLC and subsequent autoradiography. The autoradiogram showed the absence of Tp (figure 1A). This was found to be reversible, as cells grown with ammonium salt, when subcultured in the absence of ammonium salt produced Tp after a few generations. A time dependent appearance of Tp could be noted when ammonium salt-grown cells were washed, incubated with $[^{32}P]$-phosphate in a nitrogen-free medium and the tRNA isolated was analysed. It took about 6 h incubation for the detection of Tp under the experimental conditions used. On the other hand, in the case of cells previously grown in the absence of ammonium ions the presence of Tp in the tRNA could be noted in about 2 h (figure 1B).

Escherichia coli is known to contain T in its tRNA. In order to test whether the T content would change with the concentration of ammonium salt, cells were grown in a synthetic medium at two concentrations of ammonium chloride (0·49 mg and 2·8 mg N/ml) and the tRNA was analysed. The T content of the tRNA was not all affected by the ammonium ion concentration (results not presented).

Identification of the nucleotide replacing Tp

The absence of Tp in *A. vinelandii* tRNA could be due to the lack of methylation of the uridine residue at the 54th position or further modification of T to either 2′-O-methylribothymidine or to 2-thioribothymidine or to any other unknown species. In order to establish which of the different possibilities existed in the present case, RNase T_1 digest of $[^{32}P]$-labelled tRNA from cells grown under the two conditions were fractionated separately to isolate the tetranucleotide containing pseudouridine common to most tRNAs (Zamir *et al.*, 1965; Gauss and Sprinzyl, 1983). The RNase T_1 digest upon electrophoresis on DEAE-cellulose paper at pH 3·5 showed the presence of 9 bands in both the samples (figure 2A). The bands 2 and 3 in the tetranucleotide region (Sanger and Brownlee, 1967) were eluted out, digested to mononucleotides and analysed by 2-D TLC. Only band 2 from both the samples was found to contain ψp (data not shown). Contamination of band 2 by other species was obvious. Hence it was further purified by electrophoresis on Whatman 3 mm paper at pH 3·5. The autoradiogram indeed showed contamination (figure 2B). The major band (No. 4) was eluted out and analysed for its nucleotide composition.

The purified band was digested with RNase T_2 and an aliquot was subjected to electrophoresis on Whatman 3 mm paper at pH 3·5. Two spots of equal intensity at the region of Cp and Gp and a third with double the intensity at the region of ψp were
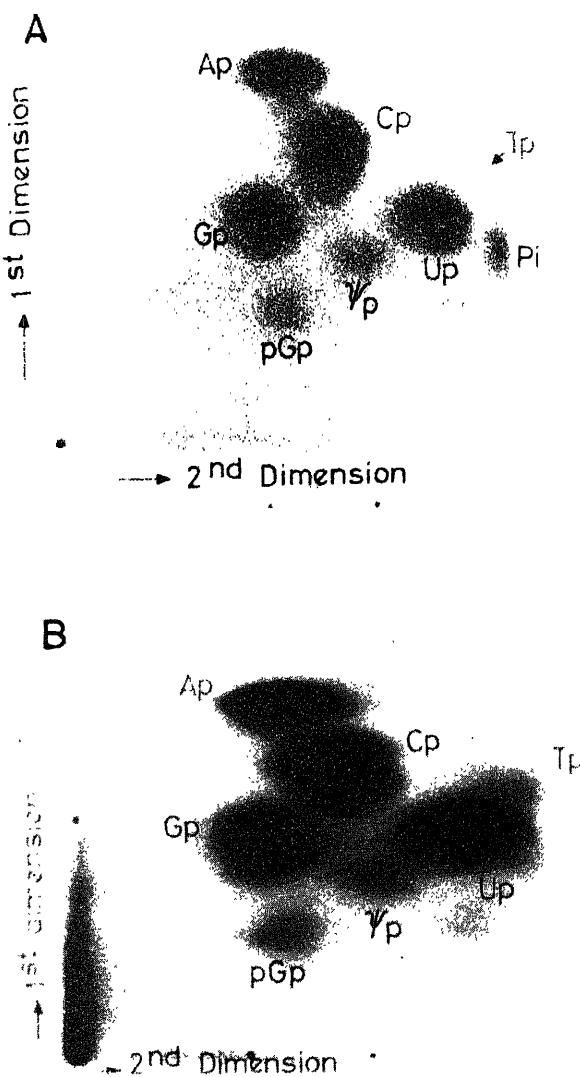
Figure 1. Autoradiogram of 2-D TLC.

RNase T_2 digest of [^{32}P]-labelled tRNA was subjected to 2-D TLC using isobutyric acid:0·5 M ammonium hydroxide, 5:3 (v/v) in the first dimension and isopropanol:conc. HCl:water, 70:15:15 (v/v/v) in the second dimension.

A. tRNA digest from *A. vinelandii* cells grown with 0·49 mg N/ml of NH$_4$Cl.

B. Cells grown in the absence of NH$_4$Cl were washed, incubated with [^{32}P]-phosphate for 2 h and the labelled tRNA was isolated and analysed.

observed in both the samples (figure 2C). Both samples had a small contamination of Ap. The radioactivity in each of the spots was measured. The relative proportion of the radioactivity in the spots was 1:1:2 (table 1). Tp and ψp move with a mobility of 0·98 with respect to Up at pH 3·5 (Smith, 1967). Therefore the ratio of radioactivity in the
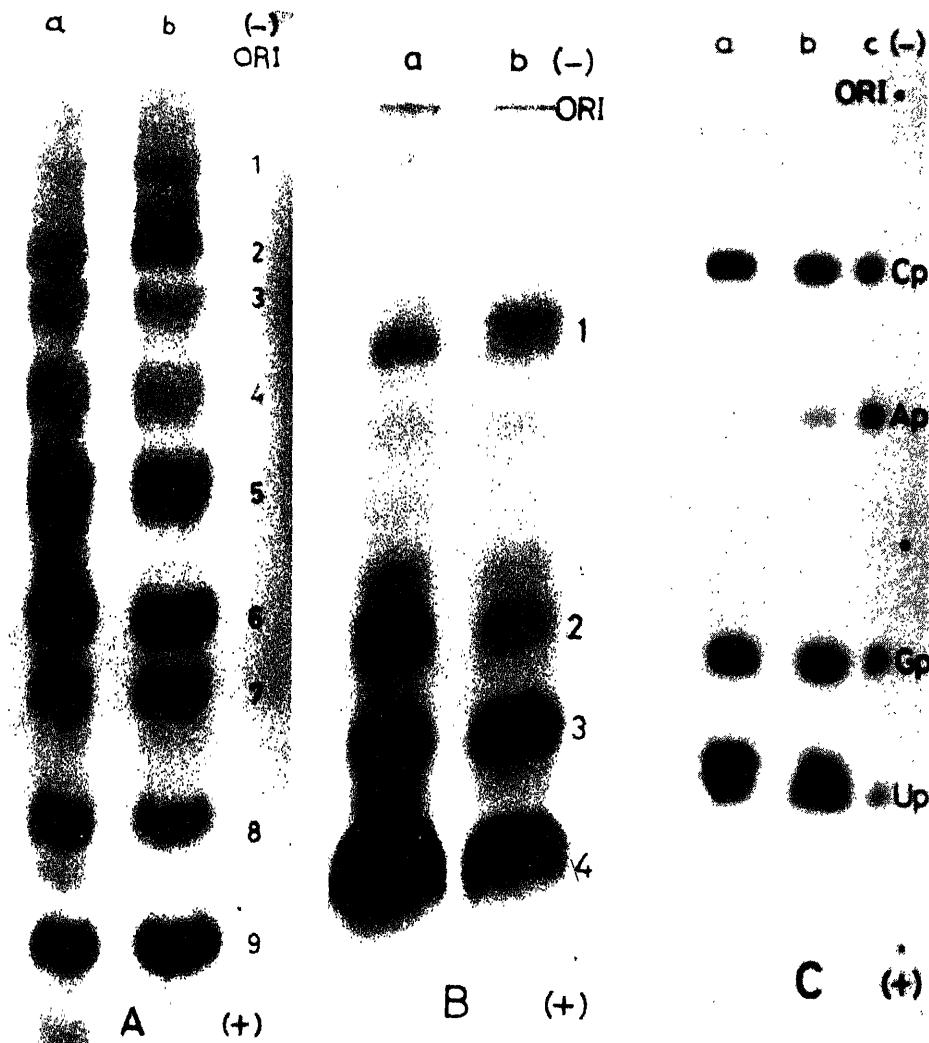
Figure 2. Analysis of the tetranucleotide fragment.

RNase T_1 digest of [^{32}P]-labelled tRNA was subjected to electrophoresis on DEAE-cellulose paper (A). The tetranucleotide (band No. 2) was eluted and subjected to re-electrophoresis on Whatman 3 mm paper (B). The purified band (the major band, No. 4) was digested with RNase T_2 and an aliquot was subjected to electrophoresis on Whatman 3 mm paper at pH 3·5 (C).

Lane a: tRNA from cells grown in the absence of NH_4^+ ions.

Lane b: tRNA from cells grown in the presence of NH_4^+ ions.

Lane c: Marker nucleotides.

spots (as in table 1) is expected for the tetranucleotide containing Tp. Since the ratio of the mononucleotides in the Tp-lacking tetranucleotide also was 1:1:2 it was evident that the spot due to the mononucleotide that replaced Tp also moved with a mobility similar to that of ψp. Amounts of radioactivity in both the samples were comparable.

Table 1. Nucleotide composition of the tetranucleotide.

Nucleotide spot	Fragment with Tp		Fragment lacking Tp	
	cpm	Ratio (Np:Gp)	cpm	Ratio (Np:Gp)
CP	21,060	0·96	15,540	0·91
Gp	21,960	1·00	17,130	1·00
Tp + ψp	44,000	2·00	—	—
Unknown + ψp	—	—	36,640	2·13

A. vinelandii cells were grown with 5 mCi of $[^{32}P]$-orthophosphate per 100 ml medium separately in the presence and in the absence of ammonium chloride and the radioactive tRNA was analysed as described in the text. The purified tetranucleotide fragment was digested with RNase T_2 and subjected to electrophoresis. Radioactive spots obtained by paper electrophoresis (figure 2C) were cut out and counted in a liquid scintillation counter.

Another aliquot of the RNase T_2 digest of the Tp-lacking tetranucleotide was mixed with non-radioactive tRNA digest and subjected to 2-D TLC. Four spots of almost equal intensity were observed. Three of them, as expected, were at the positions of Cp, Gp and ψp. The 4th coincided with the non-radioactive Up spot (figure 3). A minor spot at the region of Tp also could be noted. The radioactive spots were cut out from the plates and the radioactivity in each was determined. The amounts of radioactivity in spots were, Cp 12, 230; Gp 13, 120; Up 14, 370 and ψp 11, 650, the ratio being 0·93 : 1·00 : 1·10 : 0·89 for Cp, Gp, Up and ψp. Radioactivity in the region of Tp was only
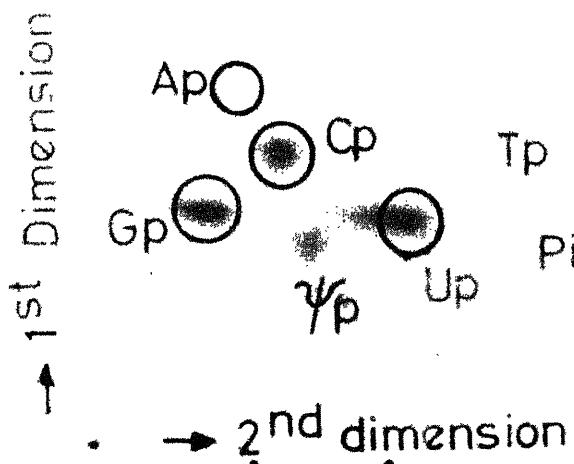
Figure 3. 2-D TLC of RNase T_2 digest of the tetranucleotide.
An aliquot of the RNase T_2 digest of the tetranucleotide (legend to figure 2) was subjected to 2-D TLC on cellulose using the solvent systems given under legend to figure 1.

390 cpm. These results established that the tRNA sample from *A. vinelandii* grown in the presence of ammonium salt had Up in the place of Tp and methylation of uridine at the 54th position had not taken place.

Tests with [^{32}P]-labelled cells washed free of ammonium salts

Non-methylation of the uridine residue of the tRNA suggested two possibilities. Either the enzyme, uridine-5-methyltransferase which methylates U to T was inhibited or the gene coding for the enzyme was repressed. If inhibition of the enzyme by the ammonium ions was involved, removal of the ammonium salt should result in methylation of the U residue to T. On the other hand, if the gene had been repressed,
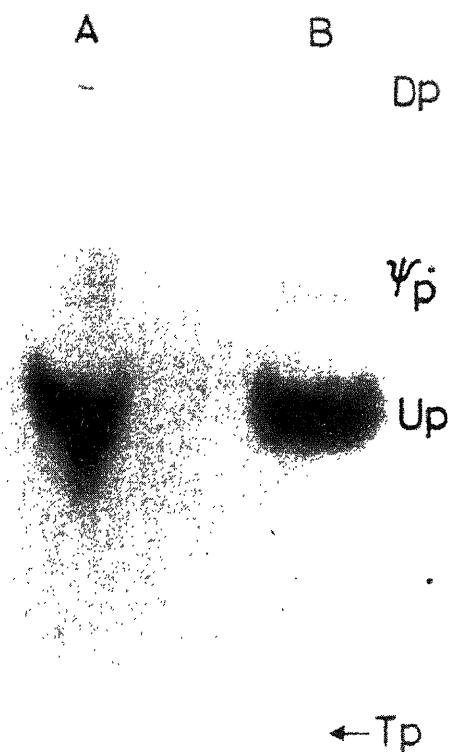
Figure 4. Analysis of RNase T_2 digest of tRNA from cells prelabelled in the presence of ammonium salt.

A. vinelandii cells grown with [^{32}P]-phosphate in the presence of ammonium salt were washed and incubated in the presence or absence of rifampicin for 2 h. tRNA isolated from the cells was digested with RNase T_2 and subjected to electrophoresis. The radioactivity from the Up region was subjected to paper chromatography in the solvent system isopropanol:water:conc. ammonia, 70:30:1 (v/v/v).
A. In the presence of rifampicin. **B.** In the absence of rifampicin.

fresh enzyme synthesis after removal of the ammonium ions would be required for the formation of T. The following experiment was carried out to distinguish between these two possibilities. *A. vinelandii* cells grown to mid-log phase with [^{32}P]-phosphate in excess of ammonium salt were washed twice with normal saline and these prelabelled cells were incubated in a nitrogen-free medium containing 100 μg/ml of rifampicin for a period of 2 h. Rifampicin addition was to prevent fresh synthesis of the enzyme. tRNA was isolated and analysed for Tp; it could not be detected (figure 4A). Prolonged incubation with rifampicin resulted in the lysis of the cells. Cells, incubated without rifampicin also did not contain Tp in the tRNA (figure 4B). However it was noticed earlier that cells previously grown in the absence of fixed nitrogen treated in a similar manner, produced Tp in the tRNA in about 2 h (figure 1B). These results suggested that the absence of Tp in the tRNA from cells grown in the presence of an ammonium salt was not due to reversible inhibition of the modifying enzyme by the ammonium ions.

Discussion

It is interesting that the tRNA of *A. vinelandii* lacks T when the organism is grown in media containing an ammonium salt. Lack of T cannot be due to any defect in the genes involved in the synthesis of T as *A. vinelandii* cells produce T when grown in the absence of ammonia. *R. meliloti*, a symbiotic nitrogen fixing bacterium also has been found to lack T in the tRNA when cultured in the presence of ammonium chloride (Ajitkumar and Cherayil, 1982). Being a symbiotic nitrogen fixer *R. meliloti* could not be cultured in the absence of fixed nitrogen. T content of *E. coli* tRNA was not at all affected by the change in the ammonium ion concentration (results not presented). It is tempting to speculate that the phenomenon of the absence of Tp in the tRNA when the organism is grown in the presence of an ammonium salt is characteristic of nitrogen-fixing bacteria. More such bacteria however, have to be studied to establish this.

Most tRNA species contain the tetranucleotide, TψCG (Zamir *et al.*, 1965). Analysis of the tetranucleotide from *A. vinelandii* tRNA has shown that it contains U instead of T when the organism is grown in the presence of ammonium ions (figures 2 and 3). Since the amount of radioactivity in the tetranucleotide was comparable in both cases (see table 1) UψCG is the prominent tetranucleotide in the tRNA from cells grown in the presence of ammonium ions. 2-D TLC analysis of the tetranucleotide digest shows the presence of a very small quantity of Tp, approximately 3 % of Gp (see text and figure 3). This suggests that even in the presence of the ammonium salt a small quantity of Tp is synthesized. This amount could not be detected in the total tRNA (figure 1A) but could be detected when it became enriched by the purification of the tetranucleotide fragment (figure 3). Much larger amount of labelled tRNA than that for 2-D TLC (figure 1) was used for the analysis of the tetranucleotide fragment (figures 2 and 3).

If the enzyme that methylates the U residue was inhibited by the NH$_4^+$ ions, removal by washing would have prevented inhibition and methylated the U residue. As the tRNA was prelabelled with [^{32}P]-phosphate formation of T would have resulted in the appearance of a radioactive spot due to Tp. Since this was not observed (figure 4) it is to be concluded that the enzyme was not present in the cells or it was irreversibly inhibited. In the case of cells previously grown in the absence of ammonium ions T is produced

under similar conditions of washing and incubation with $[^{32}P]$-phosphate (figure 1B). Under the experimental conditions used in the present studies T will be formed only if the enzyme can methylate the processed tRNA. Any precursor of tRNA would be changed to the mature species by the time the cells are harvested and washed (see above). Since formation of T is observed in one case it is to be concluded that the *A. vinelandii* enzyme, uridine-5-methyltransferase, can methylate the mature tRNA. In this respect it appears to be similar to the *E. coli* enzyme which can methylate mature tRNA$_1^{Gly}$ from wheat germ (Marcu *et al.*, 1978).

It is clear from the present results that the ammonium salt is not a reversible inhibitor of uridine-5-methyltransferase of *A. vinelandii*. However it is not clear whether it is an irreversible inhibitor or a repressor. Ammonium salt is known to be a repressor of a number of enzyme systems such as nitrogenase, glutamine synthetase and nitrate reductase (Brill, 1980; Shanmugam and Hennecke, 1979). Probably, like the nitrogenase gene uridine-5-methyltransferase gene of *A. vinelandii*, is repressed by the ammonium salt. The implications of these observations in nitrogen fixation are not clear. Modified nucleotides in tRNA are known to be involved in the expression of operons, the well known example being pseudouridine in histidine tRNA of *Salmonella typhimurium* in the expression of histidine operon (Brenner *et al.*, 1972).

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Expression of *Mycobacterium tuberculosis* genes in *Escherichia coli*

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Abstract. Two *Escherichia coli* clones expressing *Mycobacterium tuberculosis* antigens were isolated from a gene-bank in the plasmid vector pBR 325. 'Western blot' analysis revealed the presence of a unique protein band of molecular weight 68,000 and 38,000, respectively in cell-extracts from each clone. The 68,000 dalton antigen was found to be expressed on *Escherichia coli* outer surface. Plasmid DNA from a third clone could confer leucine independence on two different *leu B* mutants of *Escherichia coli* but not on mutants in other *leu* genes, pointing to the possibility of genetic complementation. Thus, *Mycobacterium tuberculosis* DNA is capable of expression in *Escherichia coli*.

Keywords. *Mycobacterium tuberculosis* gene-expression; western blot; cloned *M. tuberculosis* DNA.

Introduction

Tuberculosis remains a major health problem in the world, especially in Third World countries like India. It is believed that protective immunity against *Mycobacterium tuberculosis*, the causative organism, is generated *via* the cell-mediated immune response (Collins, 1982). The role of different subsets of T-cells and their fine specificities in tuberculosis is poorly understood. Such a study requires large quantities of relevant *M. tuberculosis* antigens in purified form—a need easily met if *M. tuberculosis* gene-products could be synthesised in *Escherichia coli*. As a first step towards this goal, we report here the expression of *M. tuberculosis* genes in *E. coli* to produce antigenically identifiable proteins.

Materials and methods

Bacterial strains and growth conditions

M. tuberculosis was grown in Middlebrook 7H9 medium containing 1 % glucose. *E. coli* strains were grown in Luria broth or in M9 medium. All cells were grown with aeration at 37°C.

Abbreviations used: PBS, Phosphate buffered saline; BSA, bovine serum albumin; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; M_r, molecular weight.

Cloning of M. tuberculosis DNA

High molecular weight DNA from *M. tuberculosis* $H_{37}R_a$ was extracted, partially digested with *EcoRI*, inserted into the *cam* site of plasmid pBR325 and cloned in *E. coli* HB101 as described before (Bhattacharya *et al.*, 1984).

Rabbit anti M. tuberculosis antiserum

M. tuberculosis $H_{37}R_v$ cells in normal saline (60 mg wet wt/ml) were sonicated for 12 min at 150 W and emulsified with incomplete Freund's adjuvant (1:1, v/v). 1·0 ml of emulsion was injected subcutaneously at multiple sites in each rabbit both for primary and subsequent boosters at two-weeks intervals. Last booster of 0·5 ml sonicate was given intravenously at the end of 8th week. Rabbits were bled every 3 weeks.

Purification of immunoglobulins and radioiodination

Immunoglobulins were purified from sera by a two-step method involving ammonium sulphate precipitation and ion-exchange chromatography as described (Mishell and Shiigi, 1980). Purified anti-bodies (10–20 μg) were radioiodinated with 200 μCi of carrier-free $Na^{125}I$ (Bhabha Atomic Research Centre, Bombay) in the presence of iodogen as previously described (Bhattacharya *et al.*, 1981).

Absorption of anti M. tuberculosis serum

1×10^6–5×10^6 cpm of $[^{125}I]$-labelled immunoglobulins, diluted to 2·0 ml with phosphate buffered saline (PBS; 10 mM phosphate, 0·14 M sodium chloride, pH 7·4) containing 1 % bovine serum albumin (BSA) were mixed with washed pellet of *E. coli* cells (100 ml cells grown to mid-log). The absorption was carried out at 4°C overnight. Unabsorbed antibodies were collected after centrifugation at 6000 *g* for 10 min at 4°C. Anti *M. tuberculosis* serum was also absorbed with sonicated *E. coli* extracts for enzyme immunoassays essentially as described (Stahl *et al.*, 1984).

Whole-cell binding assay

E. coli cells (5 ml) grown to mid-log were washed and resuspended in PBS, 1 % BSA. $[^{125}I]$-Labelled, absorbed *M. tuberculosis* antibodies (3×10^6 cpm) were added to the cells. After 2 h, incubation at room temperature with shaking, cells were washed 4 times with PBS, 0·1 % BSA. Bound radioactivity was eluted with 200 μl of 0·1 M glycine-HCl, pH 2·5. The radioactivity in the supernatant was determined using an ECIL gamma counter.

Western blotting

1–2 ml of recombinant clones grown in L-broth were centrifuged, washed with 10 mM Tris, pH 6·8, resuspended in sodium dodecyl sulphate (SDS)-sample buffer (100 μl), incubated at 100°C for 5 min and separated on 10 % polyacrylamide gels as described (Laemmli, 1970). Gels were stained with Coomassie blue and transferred electrophoretically onto a nitrocellulose sheet as described (Towbin *et al.*, 1979). Nonspecific binding sites on nitrocellulose filters were blocked with 3 % BSA in PBS

containing 2 % normal goat serum and the antigens were detected by absorbed rabbit anti *M. tuberculosis* antiserum (dil. 1:100) followed by goat anti-rabbit immunoglobulin G-horse radish peroxidase.

Results and discussion

Clones selected by antibody screening

E. coli clones from the *M. tuberculosis* gene-bank were screened using anti *M. tuberculosis* antibodies by two methods, one for clones expressing antigens on the cell-surface (screening of whole cells) and the other for intracellular antigens (screening of lysed cells). One clone each was identified by both methods and further analysed.

The clone detected by screening of whole cells was designated M-86 (cloned DNA insert size 13·8 kb). To further demonstrate the presence of *M. tuberculosis* antigens on M-86 cell-surface, a whole-cell binding assay was performed with M-86 cells and control cells (*E. coli* HB101 containing pBR325), using [^{125}I]-labelled rabbit anti *M. tuberculosis* antibodies. The results presented in table 1 show that M-86 bound significantly higher amounts of antibodies (6825 cpm) compared to control cells (392 cpm) suggesting that *M. tuberculosis* antigens are, indeed, expressed on the cell-surface of M-86 cells.

Table 1. Binding of [^{125}I]-anti *M. tuberculosis* antibodies to clone M-86 cells.

Cells	[^{125}I]-Antibody bound (cpm)
E. coli HB101 (with pBR325)	392 ± 90
M-86	6825 ± 82

The binding of [^{125}-I]-anti *M. tuberculosis* antibodies to whole cells was determined by the whole cell binding assay described in 'materials and methods'.

Further information on the nature of gene-products of M-86 responsible for the antigenic reactivity was obtained by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting of total cell-extracts from M-86 and *E. coli* containing pBR325. The results presented in figure 1 indicate the presence of a unique peptide of molecular weight (M_r) 68,000 in M-86 (lane 2). Another peptide of M_r 62,000 showed up on the western blot both in M-86 and in *E. coli* controls (lanes 1, 2). The appearance of this peptide despite exhaustive preabsorption of the rabbit antibodies with *E. coli* whole cells suggests that it is highly immunogenic and may be located intracellularly or in the inner membrane of *E. coli*.

The clone detected by screening of lysed cells was designated C-45 (DNA insert size 15 kb). On further analysis by western blotting, cell extracts from C-45 showed a major unique band of M_r 30,000 which was absent in the *E. coli* control (figure 2). These results
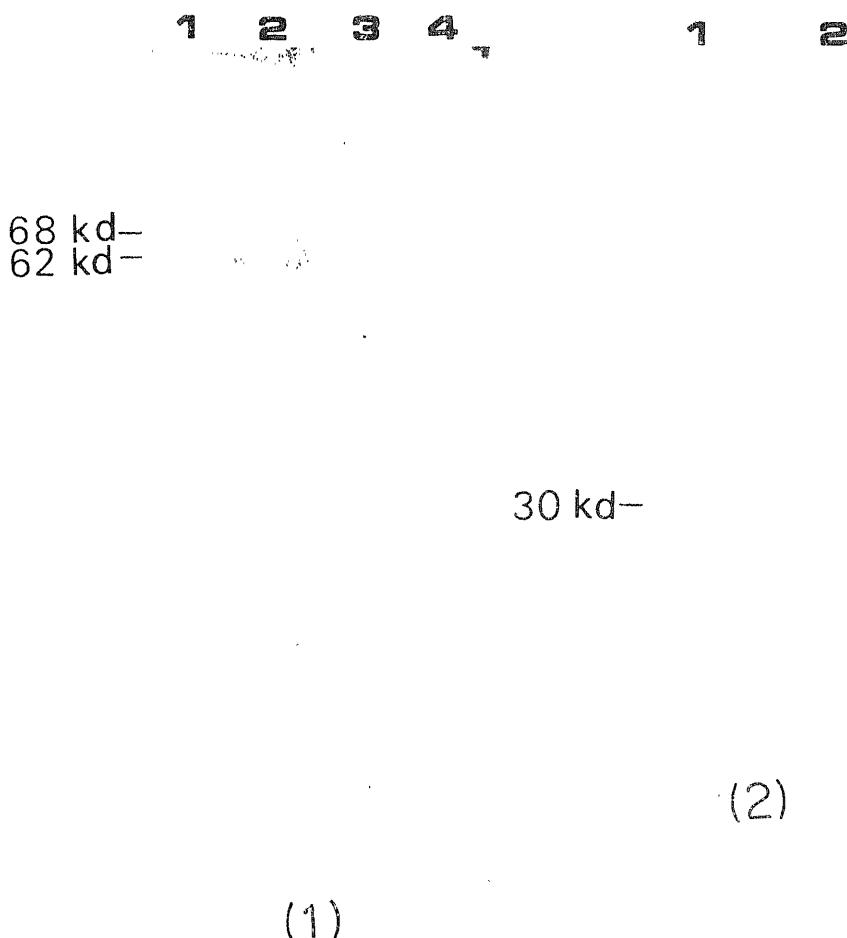
Figures 1 and 2.   1. Western blot analysis of proteins from M-86. Proteins from the clone M-86 (lanes 2, 4) and *E. coli* containing pBR325 (lanes 1, 3) were analysed by Western blotting as described in 'materials and methods'. Lanes 1, 2—blots treated with rabbit antiserum against *M. tuberculosis*. Lanes 3, 4—blots treated with preimmune rabbit serum. 2. Western blot analysis of proteins from C-45. Proteins from the clone C-45 (lane 1) and *E. coli* containing pBR325 (lane 2) were analysed by western blotting as described in 'materials and methods'. Blots were treated with rabbit antiserum against *M. tuberculosis*.

demonstrate the feasibility of expression of *M. tuberculosis* antigens from cloned DNA fragments in *E. coli*.

Clone selected by genetic complementation

E. coli HB101 carries the *leu B6* mutation which renders it auxotrophic for leucine. In order to select for clones which might complement this leucine requirement, clones growing on nutrient agar plates were replica-plated on minimal agar plates lacking leucine. One clone which on repeated sub-culturing continued to grow independent of

leucine was further studied. It was designated as L-3. It had a DNA insert size of 2·7 kb.

Leucine independence could be generated by mechanisms other than genetic complementation. To test this, plasmid DNA from L-3 (after passaging through *E. coli* DH1 to modify the DNA) was used to transform other *E. coli leu* mutants (*leuA*371, *leuB*401, *leuC*171, *leuD*101). Tiny transformant colonies came up on leu-plates for all the 4 *leu* mutants tested. When these colonies were re-tested for growth on *leu⁻* medium, it was found that only the colonies from *leuB*401 continued to grow while the others failed to grow on *leu⁻* medium. Since two different *leuB* mutants were rendered leucine independent by plasmid DNA from L-3, it seems likely that this result may be due to genetic complementation. The small amount of initial growth on *leu⁻* plates observed for all *leu* mutants may have been due to trace amounts of leucine provided by the Luria broth in which transformants were allowed to grow to enable the expression of antibiotic-resistance markers.

The expression of *M. tuberculosis* and *M. leprae* antigens in *E. coli* has recently been reported (Young *et al.*, 1985a, b) using the λ expression vector, λ gt 11. When cloning of *M. leprae* DNA was done in a cosmid vector (pHC79), no expression of mycobacterial antigens was observed (Clark-Curtiss *et al.*, 1985). However, when DNA from this library was sub-cloned into an expression vector, pYA626, polypeptides encoded by *M. leprae* DNA could be detected in minicells. Thus, all mycobacterial promoters may not be recognised by the *E. coli* transcription system. Whether they may be recognised by a gram-positive host, such as *Bacillus subtilis* remains to be investigated.

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Alteration in the metabolism of free radicals in wheat seedlings grown in the presence of 4-chloro-5-dimethylamino-2-phenyl-3(2H) pyridazinone

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Abstract. When wheat seedlings were grown in the presence of 62·5–500 μM 4 chloro-5-dimethylamino-2-phenyl-3(2H) pyridazinone, an inhibitor of photosystem II electron transport, there was a marked inhibition of *in vivo* photosystem II electron transport as revealed by the analysis of fast chlorophyll *a* fluorescence transients in intact leaves and by the inhibition (95% at 500 μM) of net photosynthesis in intact leaves. Accompanying this inhibition of photosystem II electron transport, there was a decrease in the content of photosynthetic pigments. The extent of lipid peroxidation, measured in terms of malondialdehyde content was not increased; rather it was found decreased. An analysis of *in vitro* lipid peroxidation of the thylakoid membranes of control and 4-chloro-5-dimethylamino-2-phenyl-3(2H) pyridazinone treated plants in the presence of a sensitizer dye (toluidine blue) showed a similar rate both in the control and treated samples suggesting that the availability of unsaturated fatty acids as a substrate for lipid peroxidation was not limiting even though it decreased in the treated plants. On the other hand, it appears that the availability of the free radicals for lipid peroxidation was decreased by enhanced activity of the enzyme systems involved in the metabolism of free radicals. Measurements of the activities of enzymes involved in the metabolism of free radicals showed an increase in the activities of NADPH-glutathione reductase (6–8 fold) and catalase (15–30%) and a decrease in the activity of superoxide dismutase (30–45%) in the treated plants.

Keywords. *Triticum vulgare*; free radical metabolism; BASF 13.338.

Introduction

Photosynthetic electron transport inhibitors binding at the B-protein in the vicinity of photo system II (PS II) may initiate peroxidation of membrane lipids after long-term treatment under high light intensity (Sandmann and Boger, 1982). As a consequence of inhibition of electron transport, the excited chlorophyll molecules transfer their absorbed energy to ground state oxygen yielding highly reactive singlet oxygen which in turn could undergo the process of interconversion leading to the production of hydroxyl radical and superoxide radical (Singh, 1978). Singlet oxygen is quenched by carotene found in the thylakoid membranes. Apparently, carotenoids are also broken down in this reaction. When the carotene level is decreased, photodestruction of chlorophylls as well as photoperoxidation of

Abbreviations used: PS-II, Photosystem II; BASF 13·338, 4-chloro-5-dimethylamino-2-phenyl-3(2H) pyridazinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DTT, dithiothreitol; EDTA, ethylene diaminotetraacetic acid; TCA, trichloroacetic acid; DABCO, 1,4-diazobicyclo(2-2-2) octane.

membrane lipids start (Ridley, 1977). This type of free radical mediated pigment degradation and photoperoxidation of membrane lipids is observed in cases of inhibition of photosynthetic electron transport by monuron (Pallet and Dodge, 1979) and diuron (Elstner and Oswald, 1980; Takahama and Nishimura, 1975; Ridley, 1977). All these studies were concerned with the changes in the rate of lipid peroxidation due to the addition of PS II inhibitors in the isolated chloroplasts (Takahama and Nishimura, 1975; Ridley, 1977) or mature algal cells (Elstner and Oswald, 1980) or detached cotyledons (Pallet and Dodge, 1979). To date no information is available on the status of free radical metabolism in the plants grown in the presence of PS II inhibitors.

We have been studying the changes occurring in the structure and functioning of the photosynthetic apparatus of wheat seedlings grown in the presence of 4-chloro-5-dimethylamino-2-phenyl-3(2H) pyridazinone (BASF 13.338) (Bose *et al.*, 1984; Mannan and Bose, 1985a). BASF 13.338 is a 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) type of inhibitor of photosynthetic electron transport (Mannan and Bose, 1985b). When the wheat seedlings are grown in the presence of this compound, it is taken up by the seedlings and it accumulates in the leaves to the level of inhibiting *in vivo* PS II electron transport. In spite of this inhibition of *in vivo* PS II electron transport, the BASF 13.338 treated seedlings look as healthy as the control seedlings and there is no apparent sign of free radical mediated damage. This observation suggests that certain alterations have occurred in the metabolism of free radicals resulting from the inhibition of *in vivo* PS II electron transport in the BASF 13.338 treated seedlings.

BASF 13.338 treatment has earlier been shown to interfere in the biosynthesis of linolenic acid (St. John, 1976; St. John *et al.*, 1979). Since linolenic acid is the highly preferred substrate for the free radical mediated photoperoxidative process (Boehler-Kohler *et al.*, 1981), a decrease in the content of linolenic acid could decrease the rate of free radical mediated peroxidation of membrane lipids in spite of an increase in the rate of production of free radicals through the inhibition of *in vivo* PS II electron transport. Alternatively, in the BASF 13.338 treated seedlings, there could be a stimulation in the activities of the enzymes involved in the metabolism of free radicals. With a view to examine these possibilities, we have studied in this paper the status of free radical metabolism in the BASF 13.338 treated seedlings.

Materials and methods

Cultivation of plants and BASF 13.338 treatment

Seeds of *Triticum vulgare* CV : HD 2189 were surface sterilized for 1 min with 0·1% mercuric chloride and were allowed to germinate at room temperature (30±2°C) on 3 layers of coarse filter paper in a glass Petri plate. Various concentrations of BASF 13.338 (62·5 μM, 125 μM, 250 μM and 500 μM) were prepared in 0·5% acetone and 20 ml of each solution was added initially. Thereafter, only tap water was added periodically to keep the filter paper in a moistened condition. Illumination (37·2 μE/m^2/s) was provided by a bank of 4 cool day–light fluorescent lamps (Philips, India) and a 14/10 h light/dark cycle was followed.

Determination of dry weight of the seedlings

The determination of dry weight was carried out on 10 seedlings after they had been dried for 24 h at 110°C.

Measurement of net CO_2 assimilation

The rate of photosynthetic CO_2 uptake was determined in intact leaves in an air-sealed system. The rate of CO_2 uptake was continuously monitored using an infra-red gas analyzer (Type 225, Analytical Development Co., England). Air and leaf temperature during the measurement was $30 \pm 1°C$. CO_2 concentration was approximately 350 ppm. Illumination was provided by two 100 W reflector lamps (Compatalux R40, Philips, India). The photon flux density at the surface of the leaf was 600 $\mu E/m^2$ s. To eliminate heat, a 6 cm water filter was used between light source and leaf chamber.

Isolation of chloroplast

Chloroplasts were isolated by grinding fresh leaves of *Triticum vulgare* with ice-chilled isolation medium containing 20 mM Tris-HCl buffer, pH 7·8, 5 mM $MgCl_2$, 400 mM sorbitol and 10 mM NaCl in a Sorvall Omnimixer at 50% line voltage for 10 s. The homogenate was squeezed through two layers of mira cloth and the filtrate was centrifuged for 3 min at 3500 g (Janetzki, Model K24, German Democratic Republic). The pellet obtained was washed once with the isolation medium and the final pellet containing the chloroplast was suspended to a chlorophyll concentration of 1 mg/ml.

Extraction and estimation of photosynthetic pigments

Chlorophylls and carotenoids were extracted in 80% acetone. The concentration of total chlorophyll was determined according to the method of Arnon (1949). The concentration of total carotenoids was calculated by measuring the absorbance at 480 nm after correction for chlorophyll interference (Kirk and Allen, 1965) using an extinction co-efficient of 100 $mM^{-1}cm^{-1}$ (Ridley, 1977).

Fluorescence induction

Induction of *in vivo* chlorophyll *a* fluorescence was followed in intact leaves after excitation with broad band blue light (400–520 nm, Corning 5113) at a photon flux density of 670 $\mu E/m^2/s$. The photomultiplier (Hemamatzu, R375) placed at 90° to the excitation beam was protected by an interference filter (λ_{max} 690 nm, half band width 12 nm, Schott, Federal Republic of Germany). The signal from the photomultiplier was directly displayed on a storage oscilloscope (Electronic Corporation of India Ltd., Model OS 7685).

Estimation of malondialdehyde content

Malondialdehyde content was estimated according to the method of Heath and Packer (1968). Leaf material equivalent to 200 mg fresh weight was ground in a mortar in 10 ml of distilled water and 3 ml of the preparation was incubated for 30 min with 5 ml of 0·5% thiobarbituric acid in 20% trichloro acetic acid (TCA) at

95°C. The sample was cooled, centrifuged and the optical density was measured at 532 and 600 nm. After making the correction (A532–A600) for nonspecific absorption, the level of malondialdehyde was estimated using the extinction co-efficient of 155 mM⁻¹cm⁻¹

In vitro lipid peroxidation

The reaction mixture for the study of *in vitro* lipid peroxidation in a final volume of 3 ml contained (unless otherwise stated): 10 mM Tris-HCl pH 7·5 and chloroplast membranes equivalent to 60 μg chlorophyll/ml. The reaction mixture was under continuous stirring and the temperature was maintained at 30°C. Irradiation was provided at the photon flux density of 148·8 μE/m²/s. Toluidine blue (photosensitizer), 1,4-diazobicyclo(2-2-2) octane (DABCO) and β-carotene (singlet oxygen quenchers) were used at the concentration of 30 μM, 10 mM and 5 μM, respectively. After 30 min of incubation, the content of malondialdehyde was estimated.

Extraction of enzymes from leaves

Fully expanded primary and secondary leaves were removed from the seedlings and used immediately. Leaves were cut into small bits and homogenized with a cold mortar and pestle. The grinding medium contained 50 mM Tris-HCl, pH 7·8, 10 mM MgCl₂, 5 mM dithiothreitol (DTT) or 10 mM β-mercaptoethanol and 0·25 mM ethylene diamino tetra acetic acid (EDTA). The extract was clarified by centrifugation for 10 min at 20000 g (Sorvall, Model RC-5B, USA). The clear supernatant was decanted slowly and used as the enzyme source. All the procedures were carried out at 4°C and the assays were completed within 1 h.

Enzyme assay

Catalase was assayed by measuring the initial rate of disappearance of hydrogen peroxide by the method of Chance and Machly (1955). Superoxide diamutase was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium using the method of Beauchamp and Fridovich (1971). Glutathione reductase was assayed according to Schaedle and Bassham (1977) by monitoring glutathione dependent oxidation of NADPH at 340 nm.

Results

When wheat seedlings were grown in the presence of various concentrations of BASF 13.338, there was a small decrease in the growth rate as shown by a reduction in the dry weight of the seedlings (table 1). There was also a decrease (20%) in the content of photosynthetic pigments on fresh weight basis (table 2) and in the rate of net CO₂ assimilation.

When the dark incubated leaves were illuminated there was a transient increase in the level of chlorophyll *a* fluorescence emission to a maximum level within a few hundred ms. A faster rise to the maximum level in such a chlorophyll *a* fluorescence transient indicates an inhibition in the photosynthetic electron transport chain between the primary and secondary electron acceptors of

Table 1. Changes in the dry weight and rate of net CO_2 assimilation in the wheat seedlings due to treatment with BASF 13.338.

BASF 13.338 concentration (μM)	Dry weight (mg/10 seedlings)	Net CO_2 assimilation mg $CO_2/m^2/s$
0·0	181·0	0·267
62·5	170·5	0·210
125·0	160·4	0·064
250·0	145·3	0·028
500·0	120·8	0·014

Measurements were made with 12 days old seedlings.

Table 2. Changes in the content of chlorophylls, carotenoids and malondialdehyde in 12 days old wheat seedlings due to treatment with BASF 13.338.

BASF 13.338 concentration (μM)	Chlorophyll (mg/gram fresh wt.)	Carotenoids (μmol/g fresh wt.)	Malondialdehyde (nmol/g fresh wt.)
0·0	1·95	0·98	355·7
62·5	1·79	0·85	241·7
125·0	1·70	0·81	203·4
250·0	1·65	0·81	214·8
500·0	1·58	0·77	179·6

photosystem I (Mannan and Bose, 1985b). An analysis of such fast fluorescence transient of chlorophyll *a* in the intact leaves showed a faster rise of fluorescence intensity to the maximum level (F_m) in the BASF 13.338 treated plants indicating a decrease in the rate of reoxidation of primary acceptor of PS II (figure 1). This inhibition of *in vivo* photosystem II electron transport is in agreement with the observed decrease in the rate of net CO_2 assimilation in the BASF 13.338 treated wheat seedlings (table 1).

In spite of this notable inhibition of *in vivo* photosynthetic electron transport in the BASF 13.338 treated plants, the extent of free radical mediated lipid peroxidation was lower when compared to that of control seedlings. The rate of free radical mediated lipid peroxidation is usually estimated by measuring the formation of end products, *viz.* malondialdehyde or ethane (Heath and Packer, 1968; Elstner and Oswald, 1980). In our *in vivo* system, estimation of malondialdehyde was convenient. Table 2 shows that the content of malondialdehyde decreased with the increasing concentrations of BASF 13.338 treatment.

The results of *in vitro* lipid peroxidation experiments are shown in table 3. In this experiment, the thylakoid membranes isolated from control and BASF 13.338 treated plants were illuminated in the presence of a sensitizer dye toluidine blue, which is widely used as a sensitizer dye in *in vitro* lipid peroxidation studies (Anderson and Krinsky, 1973). When toluidine blue is illuminated with visible light, singlet oxygen is formed which can peroxidatively damage the membrane
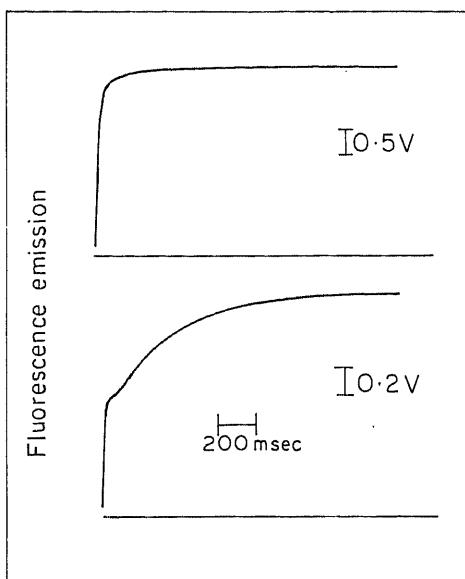
Figure 1. Fast fluorescence transients of chlorophyll *a* in the intact leaves of control (lower) and BASF 13.338 (125 μM) treated plants (upper).

Table 3.   Results of the *in vitro* lipid peroxidation experiments involving thylakoid membranes isolated from control and BASF 13.338 (250 μM) treated wheat seedlings.

		Malondialdehyde production			
		nmol/mg chlorophyll		nmol/mg total lipids	
		Control	Treated	Control	Treated
No addition	Dark	23·66	17·36	2·29	1·88
No addition	Light	44·10	48·45	4·27	5·04
+Toluidine blue	Dark	27·36	21·58	2·65	2·34
+Toluidine blue	Light	75·43	83·84	7·31	9·09
+Toluidine blue +DABCO	Light	19·59	22·05	1·90	2·39
+Toluidine blue + β carotene	Light	26·61	26·74	2·58	2·90

lipids in the surrounding medium. The rate of toluidine blue mediated lipid peroxidation is measured in terms of the rate of production of malondialdehyde and is sensitive to the well known singlet oxygen quenchers such as DABCO and β-carotene.

When the thylakoid membranes isolated from the control and BASF 13.338 treated wheat seedlings were kept under illumination there was an increase in the rate of lipid peroxidation as measured from the rate of production of malondialdehyde over that of dark incubated sample. This light mediated increase in the rate of lipid peroxidation was further enhanced in the presence of toluidine blue. Both

the light mediated and toluidine blue enhanced rates of lipid peroxidation in the isolated thylakoid membranes were sensitive to both DABCO and β-carotene. The interesting and significant difference between the control and treated plants was that there was no significant difference between the control and treated plants in the rate of *in vitro* lipid peroxidation both on chlorophyll basis and lipid basis. This observation was in contrast to the situation *in vivo* where the treated plants showed a definite decrease in the rate of lipid peroxidation.

Superoxide dimutase, glutathione reductase and catalase are thought to be the enzymes involved in the metabolism of free radicals in plant systems (Halliwell, 1978). Table 4 shows that compared to the control plants, the BASF 13.338 treated plants exhibited a considerable increase in the activity of both catalase and NADPH-glutathione reductase. Thus in the wheat seedlings, grown in the presence of 250 μM BASF 13.338, there was about 25% increase in the activity of catalase and about 5–6 fold increase in the activity of NADPH-glutathione reductase. However, superoxide dismutase responsible for the dismutation of superoxide was found decreased in the treated plants.

Table 4. Changes in the content of catalase, NADP-glutathione reductase and superoxide dismutase in 12 days old wheat seedlings due to treatment with BASF 13.338 (250 μM).

Enzyme	Control	Treated
Catalase	210×10^3	255×10^3
	255×10^3	278×10^3
	182×10^3	234×10^3
	137×10^3	428×10^3
NADP-glutathione reductase	4·8	31·2
	8·1	47·7
	3·1	24·4
	4·6	28·7
Superoxide dismutase	28·6	16·0
	13·8	9·9
	39·3	21·3
	14·0	8·5

The values are given as unit/mg protein. Results from 4 different experiments have been given.

Discussion

When the wheat seedlings are grown in the presence of BASF 13.338, this compound is taken up by the plants and is accumulated in the leaves to the level of inhibiting *in vivo* photosynthetic electron transport (figure 1). The inhibition of *in vivo* photosynthetic electron transport (figure 1). The inhibition of *in vivo* photosynthetic electron transport is expected to induce an increase in the rate of production of free radicals leading to an increase in the rate of peroxidation of membrane lipids. Contrary to this expectation, the extent of lipid peroxidation measured in terms of malondialdehyde content decreased considerably in the treated plants (table 2). A possible mechanism for the observed decrease in the extent of lipid peroxidation in the BASF 13.338 plants has been discussed below.

One possible explanation is that BASF 13.338 induced alterations in the fatty acid composition of the membrane lipids (St. John, 1976; St. John *et at.*, 1979) might result in a decrease in the rate of lipid peroxidation. But *in vitro* lipid peroxidation experiments with the isolated thylakoid membranes in the presence of a sensitizer dye showed that the rate of lipid peroxidation was similar both in the control and BASF 13.338 treated samples (table 3). This observation indicates that the availability of unsaturated fatty acids as a substrate of lipid peroxidation was not limiting even though it decreased in the treated plants. So it appears that the availability of free radicals mediating the lipid peroxidation could have been decreased by the enhanced activity of the enzyme systems involved in the metabolism of free radicals.

In the BASF 13.338 treated plants, NADPH-glutathione reductase activity increased 8-fold. This enzyme has been reported to be present in the chloroplasts (Schaedle and Bassham, 1977) and its role in the protective reactions against photooxidative damage is well known (Halliwell, 1978; Forster and Hess, 1980). Like NADPH-glutathione reductase, the activity of catalase was also increased in the BASF 13.338 treated plants. Catalase is localized in the leaf microbodies which are generally considered as safety valves against the photoperoxidative damage (Feierabend and Schubert, 1978).

In contrast to the increase in the activities of catalase and NADPH-glutathione reductase, the superoxide dismutase showed a decrease in its activity in the BASF 13.338 treated plants. In higher plants, superoxide dismutase functions in the metabolism of superoxide (Halliwell, 1978; Dhindsa and Matowe, 1981). Normally, in plants, the superoxide radical production is believed to result from the operation of pseudocyclic electron transport (Egneus *et al.*, 1975; Ivanov and Povalyalva, 1979). Inhibition of *in vivo* non-cyclic electron transport due to BASF 13.338 treatment is expected to cause a decrease in the pseudocyclic electron transport leading to a decrease in the production of superoxide radicals. Since superoxide dismutase is an inducible enzyme (Gregory and Fridorich, 1973; Abeliovich *et al.*, 1974), the reduction in the production of superoxide radical could ultimately account for the observed decrease in the activity of superoxide dismutase in the BASF 13.338 treated plants.

Acknowledgement

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Mitochondrial functions during experimentally induced cardiac hypertrophy

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Abstract. When the hearts of albino rats are subjected to pressure-induced stress through constriction of ascending aorta, changes in the mitochondrial functions are observed as early as 24 h after the imposition of the stress. These include the abolition of oxidative phosphorylation, decrease in the energy dependent [^{45}Ca]-uptake and decrease in the rate of energized swelling. A large influx of calcium ions and an increase in the fluidity of mitochondrial membranes also occur in this period. At later stages of hypertrophy (17, 28, 40%), these mitochondrial functions gradually return to normal levels.

Keywords. Cardiac hypertrophy; mitochondria; oxidative phosphorylation; respiratory control index; Arrhenius plot; energized swelling.

Introduction

Mitochondria of various tissues respond differentially to different types of stress with regard to the bioenergetic functions such as oxidative phosphorylation, ion uptake and energized swelling. When freshwater fishes are subjected to salinity stress, the muscle and gill mitochondrial energy functions are altered in an early response phase (Bashyam *et al.*, 1980; Suresh and Jayaraman, 1983). No such changes are observed in the mitochondria isolated from hearts of rats (Davies *et al.*, 1981) or dogs (Wollenberger *et al.*, 1961) when subjected to endurance training and gradually induced aortic stenosis, respectively.

As myocardial metabolism is almost exclusively aerobic, the energy required for muscle contraction, ion transport, rhythmicity and for the synthesis of membrane and protein constituents of myocardium are solely supplied by mitochondrial oxidative phosphorylation. In the present system of experimentally induced cardiac hypertrophy in rats produced by constricting the ascending aorta, we have investigated the biogenesis and energetics of mitochondria and the regulatory mechanism governing the process during cardiac hypertrophy. Earlier studies have shown an increase in the mitochondrial biogenesis as early as 24 h after imposition of work load through aortic constriction (Thirunavukkarasu *et al.*, 1982). Our results in the present study show an initial response phase at 24 h stage characterized by the altered mitochondrial functions which are restored to normal levels gradually at the later stages of hypertrophy.

Materials and methods

Female albino rats of Wistar derived strain (obtained from Rallis India, Bangalore) were used for the experiments. Animals (150–180 g) were allowed one week to acclimatize to local conditions before aortic constriction was done using a small hemostatic clip (Hemoclip Edward Weck and Company, North Carolina, USA). The animals were fed with Gold Mohur rat feed received from M/s Hindustan Lever Limited, Bangalore, India.

Chemicals

Most of the chemicals used in the experiments were of analar grade obtained from British Drug House and E. Merck, Darmstadt, Germany. Fine chemicals such as adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP) etc. were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. $^{45}CaCl_2$ was purchased from the Bhabha Atomic Research Centre, Bombay.

Induction of cardiac hypertrophy

Cardiac hypertrophy was induced by following the method of Rakuson and Poupa (1966) and the percentage hypertrophy was calculated as the percentage increase in the heart weight/body weight ratio of hypertrophic animals over that of the ratio of sham-operated controls (Meenakshi *et al.*, 1983).

Isolation of heart mitochondria

Isolation of mitochondria was carried out in a medium containing 250 mM sucrose, 10 mM Tris-HCl, pH 7·5 and 1 mM EDTA. The hearts were homogenized in the isolation medium in a ratio of 1:10. The mitochondria were isolated as described in the earlier paper (Thirunavukkarasu *et al.*, 1982). For $^{45}CaCl_2$ uptake and endogenous ion estimation, the mitochondrial pellet was washed 3 times with an EDTA-free isolation medium.

Analytical methods

Protein was estimated according to Lowry *et al.* (1951) using bovine serum albumin as the standard. Oxygen consumption was measured by polarography (Estabrook, 1967) with a clark type oxygen electrode attached to a recorder. The respiratory control index (RCI) was calculated as the ratio of the rate of oxygen consumption in the presence of ADP (state III) to the rate after the added ADP is completely utilized (state IV). ADP/O ratio was calculated as described earlier (Chance and Williams, 1955).

Endogenous ionic levels

The mitochondrial pellet was washed with EDTA-free isolation medium prepared in deionized and double distilled water. The washed pellet was suspended in 0·05 N HCl. The suspension was kept in boiling water for 30 min and then centrifuged at 10,000 *g*. The clear supernatant fraction was suitably diluted and the ions Na^+ and Ca^{2+} were estimated by flame photometry.

Energy dependent calcium uptake

The procedure adopted for energy-dependent Ca^{2+} uptake by mitochondria was as described by Loyter *et al.*, (1969). The mitochondrial pellet, after washing twice with cold EDTA free medium, was suspended at a concentration of 0·5 mg/ml in the assay medium which contained 150 mM KCl, 4 mM potassium phosphate buffer (pH 7·4), 5 mM Tris-HCl (pH 7·4), 4 mM $MgSO_4$, 5 mM ATP and 10 mM succinate. The reaction was initiated by $^{45}CaCl_2$ and after incubation for 10 min at 30°C, the reaction was stopped by adding 0·1 ml of 400 mM $MgSO_4$. The samples were pelleted by centrifuging at 10,000 g for 2 min in a Janetzki T.H. 12 model. After washing the pellet twice with cold assay medium, the pellets were dissolved in 0·1 N NaOH. These samples were then spotted on Whatman No 1 filter paper discs, dried and counted in a liquid scintillation counter.

Mg^{2+}-dependent ATPase

Mg^{2+}-dependent ATPase activity was measured in the mitochondrial preparation according to the procedure of Tzagoloff (1970). The activity was assayed at 30°C in a total volume of 1 ml containing 50 mM Tris-HCl, pH 8·5, 4 mM ATP, 5 mM $MgSO_4$ and mitochondrial protein of about 70 μg as the enzyme source. The reaction was initiated by the addition of ATP, stopped after 10 min by the addition of 0·5 ml of 10% tricarboxylic acid (TCA), and the tubes were kept at 0°C for 15 min. After pelleting down the precipitate the supernatant fraction was assayed for orthophosphate by the method of Fiske and Subba Row (1952).

Density gradient analysis

For the density gradient analysis a discontinuous sucrose gradient of 30, 35, 40, 45, 50, 60, 65 and 70% (w/v) sucrose was used as described earlier (Suresh and Jayaraman, 1983). Succinate dehydrogenase activity and protein estimations were made in the fractions collected after centrifugation.

Energized swelling

The high amplitude energized swelling of the mitochondria was measured according to the method of Dow *et al.* (1970). Heart tissue was homogenized and the isolation of mitochondria was carried out using a medium containing 210 mM mannitol, 70 mM sucrose and 1 mM EDTA. About 100 μg mitochondria was added to 1 ml of medium containing 2 mM sodium acetate, 110 mM sodium chloride, 2·5 mM Tris-HCl, pH 7·4, 35 mM mannitol and 0·5 mM EDTA. Swelling was measured as a decrease in absorbance at 520 nm on addition of ATP.

Miscellaneous

The cholesterol content of mitochondria was estimated according to the method of Abell *et al.* (1952) by using Liebermann-Buchard reagent. Extraction of total lipids was carried out following the procedure of Bligh and Dyer (1959). The method of King and Wooton (1959) was followed for the estimation of lipid phosphorus.

Results

Oxidative phosphorylation

A significant increase in succinate oxidation was observed in mitochondria isolated from animals at 24 h after aortic constriction as compared to that of sham-operated. There was no significant change in activity in the mitochondria obtained from animals as early as 6 h after operation or at later stages later than 24 h during developing cardiac hypertrophy. At 24 h post-operative stage the mitochondria exhibited a loss in the ability to phosphorylate the externally added ADP as measured by ADP/O ratio. This function is restored in the mitochondria isolated from hearts at 17, 28 and 40% hypertrophic stages (table 1).

Table 1. Respiratory status of the mitochondria *in vitro* in response to aortic constriction.

Mitochondria	n atoms oxygen uptake/ min/mg mitochondrial protein	Respiratory control index	Mg^{2+}-dependent ATPase levels (μmol P_i/min/mg protein)
	succinate oxidation		
Sham operated	150 ± 30	4·6	$0·63 \pm 17$
6 h after operation	162 ± 12	4·2	$0·57 \pm 26$
24 h after operation	234 ± 42	1·0	$0·32 \pm 09$
17% hypertrophy (2)	174 ± 24	4·0	$0·61 \pm 12$
28% hypertrophy (4)	156 ± 12	4·3	$0·69 \pm 18$
40% hypertrophy (8)	168 ± 12	4·4	$0·86 \pm 21$

2 mg mitochondrial proteins were added to the incubation buffer. 10 mM succinate and 300 nmol of ADP were added to induce respiration. The values in parentheses refer to the number of days after the aortic constriction. The values presented in the table represent the mean of 3 different experiments with ±S.D.

Mg^{2+}-dependent ATPase activity

In order to check whether any alteration occurs in the specific activity of the enzyme located in the inner mitochondrial membrane, Mg^{2+}-dependent ATPase activity was estimated in mitochondria isolated from sham-operated hearts and hearts at different stages of hypertrophy. A slight increase at 6 h post-operative stage and a 2-fold decrease at 24 h after operation in the ATPase activity as compared to that of sham-operated controls were observed. The enzyme activity returned to that of normal values in the later stages of hypertrophy (table 1).

Density gradient analysis

The homogeneity of the mitochondrial preparations from hearts of sham-operated, 24 h post-operative stage and 40% hypertrophic animals, was checked by sucrose density gradient analysis. The mitochondria isolated from hearts of sham-operated and 40% hypertrophic animals showed a major band at 1·2025 g/cm^3. However, the mitochondria from hearts of animals at 24 h post-operative period showed a major band at 1·2296 g/cm^3 and a minor one at 1·2025 g/cm^3 (figure 1).
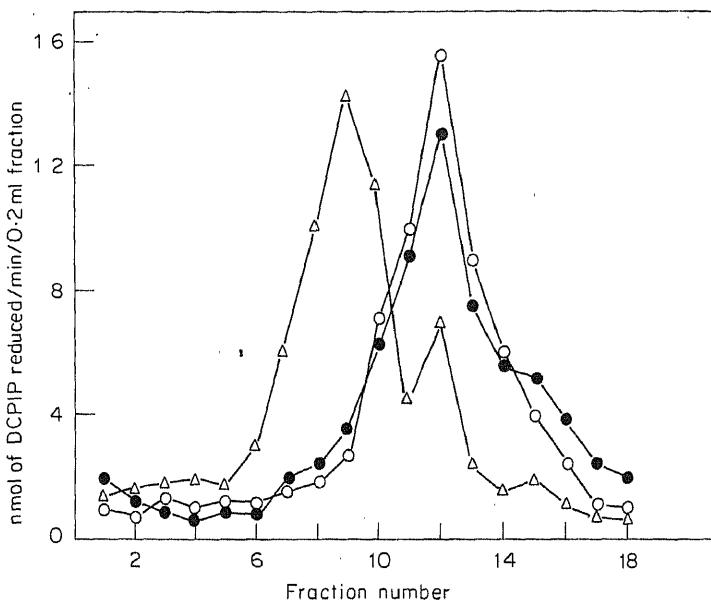
Figure 1. Sucrose density gradient analysis of mitochondria (70–30%).
(○), Mitochondria from sham-operated; (△), mitochondria isolated from 24 h after operation; (●), mitochondria from 40% hypertrophic animals.

Energized swelling and cholesterol to phospholipid ratio

A 3-fold decrease in the energized swelling and a 2-fold decrease in cholesterol to phospholipid ratio was observed at 24 h after operation when compared to that of sham-operated controls (table 2). The mitochondria isolated from animals at 6 h after operation did not change very much in the cholesterol to phospholipid ratio but a significant decrease in the energized swelling was seen as compared to the

Table 2. Quantitative analysis of mitochondrial membrane components and energized swelling rate after aortic constriction.

Mitochondria	Lipid/protein	Cholesterol/phospholipid	Energized swelling Δabsorbance/ min/100 μg mitochondrial protein
Sham operated	0·30±0·09	0.08±0·002	0·107±0·023
16 h after operation	0·25±0·03	0·07±0·004	0.060±0·007
24 h after operation	0.25±0·02	0·04±0·006	0.035±0·003
17% hypertrophy (2)	0·26±0·01	0·06±0·002	0·060±0·009
28% hypertrophy (4)	0·28±0·04	0·08±0·001	0·070±0·013
40% hypertrophy (8)	0·26±0·02	0·08±0·002	0·070±0·015

The values in parentheses refer to the number of days after aortic constriction. The values presented in the table are the mean of 3 different experiments with ±S.D.

sham-operated controls. The decrease in energized swelling and the decrease in cholesterol to phospholipid ratio at 24 h after operation suggested that the mitochondria may be already in a swollen state and the energized uptake of calcium ions may be interfered with.

Endogenous levels of ions and uptake of Ca^{2+}

A 3-fold decrease in the Ca^{2+} uptake was seen in mitochondria isolated from animals at 24 h after operation as compared to the sham-operated and the uptake reached that of normal level at later stages of hypertrophy. The decrease in Ca^{2+} uptake is significant as early as 6 h post-operative stage itself though a maximum level of decrease is seen only at the 24 h after operatioh (table 3). The 60% increase

Table 3. Endogenous levels of calcium and sodium ions and $^{45}Ca^{2+}$ uptake after aortic constriction.

Mitochondria	Endogenous levels of ions ng/mg mitochondrial protein		$^{45}Ca^{2+}$ uptake nmol/mg mitochondrial protein/min	$^{45}Ca^{2+}$ uptake (%)
	Sodium	Calcium		
Sham operated	29·4±2·3	32·3±2·8	11·3	14·1
6 h after operation	25·5±1·9	39·6±3·1	8·1	10·1
24 h after operation	17·3±0·9	51·1±4·7	4·2	5·3
17% hypertrophy (2)	22·8±0·2	42·2±3·4	9·9	12·3
28% hypertrophy (4)	29·1±2·0	36·8±2·9	9·5	11·9
40% hypertrophy (6)	33·6±2·5	34·1±2·6	10·1	12·6

80 nmol of $^{45}Ca^{2+}$ was added to the reaction mixture to start the reaction for studying the Ca^{2+} uptake. The conditions are as described in the methods section. The values in the parentheses refer to the number of days after aortic constriction. The values presented in the table are the mean of 3 different experiments ±S.D.

in the endogenous calcium levels was accompanied by a 50% decrease in the endogenous sodium levels in the mitochondria obtained from animals at 24 h after operation when compared to that of sham-operated controls. However, the levels of both ions reached that of sham-operated controls gradually in the 17, 28 and 40% hypertrophy (table 3). A slight decrease in endogenous sodium and an increase in the calcium levels were observed in mitochondria at 6 h post-operative stage. The results presented in figure 2 shows an inverse correlation (correlation coefficient −0.9) between energy dependent ^{45}Ca uptake and the endogenous levels of calcium. These results in addition to the decrease in energized swelling clearly show that alterations in the mitochondrial membrane functions have occurred and these alterations may be reflected in the fluidity of the membrane.

Arrhenius plots

The Arrhenius plots for Mg^{2+}-dependent ATPase activity representing the fluidity of the mitochondrial membranes in the mitochondria isolated from sham-operated hearts and hearts from 24 h post-operative stage and 40% hypertrophy are presented in figure 3. At 24 h after operation, the transition temperature of 18°C,
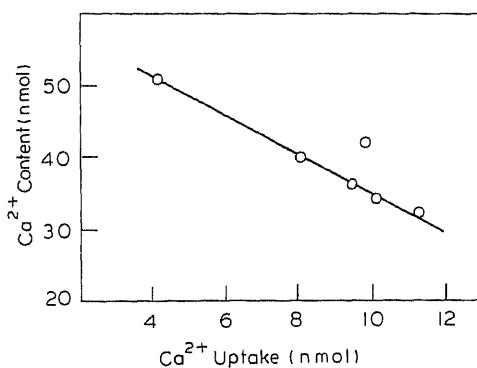
Figure 2. Correlation curve of Ca^{2+} uptake and Ca^{2+} content.

The uptake of $^{45}Ca^{2+}$ and the estimation of calcium content were as given under 'materials and methods' section. The correlation coefficient is -0.9.
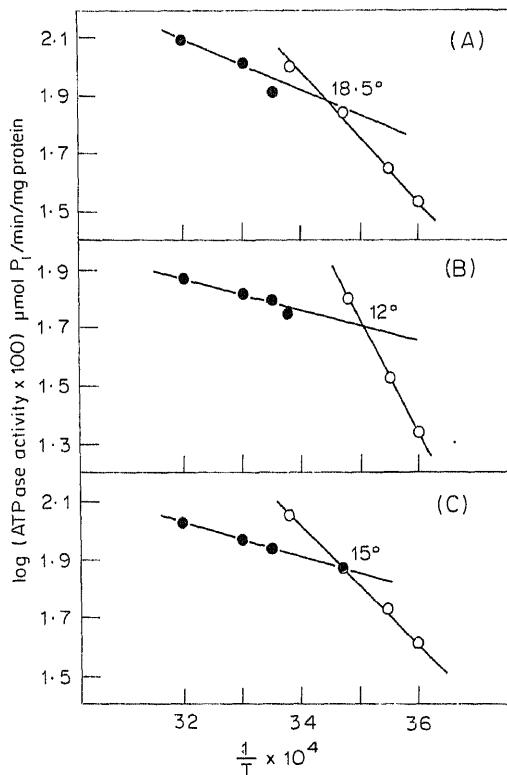
Figure 3. Arrhenius plots of mitochondrial Mg^{2+} dependent ATPase enzyme activity. A. Sham operated controls. B. 24 h after operation. C. 40% hypertrophy.

The transition temperature of the phase change is given in the figure. The activation energy values above the transition temperature are 4·6, 3·2 and 5·4 Kcal/mol in A, B and C, respectively. The activation energy values below the transient temperature are 8·7, 12·5 and 6·5 Kcal/mol in A, B and C, respectively.

observed in mitochondria from sham-operated animals, decreased to 12°C, while during 40% hypertrophic stage, it increased to 15°C tending towards the value of sham-operated controls. The activation energy above the transition temperature decreased from 4·6 Kcal/mol in sham-operated to 3·2 Kcal/mol at 24 h after operation and increased to 5·39 Kcal/mol in the 40% hypertrophic stage (figure 3). A reverse trend was seen in the activation energy values below the transient temperature.

Discussion

Calcium is considered to play an important role in the control of metabolic events between mitochondria and cytoplasm in normal cells (Bygrave, 1978). Several reports have attributed the decreased ADP/O ratio, and respiratory control index to the increased loading of calcium in the mitochondria (Rossi and Lehninger, 1964; Villalobo and Lehninger, 1980). Under high salinity stress in fish muscles and gills (Bashyam *et al.*, 1980; Suresh and Jayaraman, 1983) and during muscular dystrophy in hamsters (Mezon *et al.*, 1974), mitochondrial accumulation of calcium ions leads to loss of ADP/O ratio, respiratory control index and uncoupler stimulated respiration.

Our results presented here show a decrease in respiratory control index, ADP/O ratio accompanied by a decreased uptake of calcium ions in mitochondria obtained from aorta-constricted rats at 24 h after operation. A 60% increase in endogenous calcium levels observed at 24 h after aortic constriction may be responsible for the decreased uptake of calcium and associated changes. This view is further strengthened as the respiratory functions and calcium uptake efficiency return to normal levels as the endogenous calcium levels reach that of sham-operated controls at later time points. The shift in the peak of SDH activity to the heavier density region in the density gradient analysis of mitochondria obtained from animals at 24 h after operation as compared to that from sham and 40% hypertrophic animals could be attributed to the increased endogenous calcium levels (figure 1 and table 2).

Influx of calcium ions has been suggested to cause swelling of mitochondria (Carafoli and Lehninger, 1964; Drahota *et al.*, 1965). It remains to be shown whether this reason could be attributed to the decreased energized swelling we have observed in mitochondria from animals at 6 h and 24 h after operation. As mitochondria from sham-operated animals and from hearts at later stages of hypertrophy have lower endogenous levels of calcium ions as compared to that of 24 h stage, the capacity for energized swelling is retained in these mitochondria. The drastic decrease in cholesterol to phospholipid ratio and the corresponding increase in the fluidity of mitochondrial membranes at 24 h post-operative stage could also have contributed to the calcium loading of mitochondria causing decreased swelling at 24 h stage.

Changes in lipid composition of erythrocyte ghosts leading to changes in permeability have been reported in muscular dystrophy (Howland and Iyer, 1977). Also, in ischemia, the liver mitochondria were found to be swollen with loss of respiratory control due to an altered lipid composition (Mittnacht *et al.*, 1979). Wollenberger and Schulze (1961) have reported ultra-structural evidence of a

mitochondrial defect in chronically overloaded myocardium of dogs. This information along with our results presented in this paper led us to the presumption that although the stress was caused by different factors, the response by mitochondria usually involved an altered lipid composition leading to a change in membrane configuration. Such a change could cause an influx of calcium into mitochondria resulting in decreased respiratory functions. The transient decrease in the bioenergetic functions during the initial phase of stress and restoration to normalcy later indicate gradual adaptation in the mitochondrial functions at the molecular level to meet the challenge thrown by the imposed stress.

Acknowledgements

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Iodide transport in isolated cells of mouse submaxillary gland

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Abstract. A method has been developed to isolate cells from the submaxillary gland of mouse by treatment with pronase. Three fractions of cells have been isolated having almost equal iodide concentrating activity. The isolated cells show time dependent uphill transport of iodide. The transport is substrate-saturable, having a K_m value of 0·3 μM for iodide. The transport is sensitive to antithyroid drugs, metabolic inhibitors and to some extent to ouabain. Pseudohalide such as thiocyanate competes with the transport of iodide. Thyroid hormones or thyroid stimulating hormone have no significant effect on the iodide transport in these cells.

Keywords. Iodide transport; iodide concentration; isolated cells of submaxillary gland.

Introduction

It is known for a long time that apart from the thyroid gland, iodide ion is concentrated in the salivary gland of mouse (Fletcher *et al.*, 1959; Banerjee and Datta, 1982), in saliva of a number of other species (Cohen and Myant, 1959; Honour *et al.*, 1952), in mammary tissue and in milk (Brown-Grant 1961; Freinkel and Ingbar, 1956) and also in the stomach and its secretion (Banerjee and Datta, 1982; Honour *et al.*, 1952). However, studying the mechanism of iodide transport in these extrathyroidal tissues by *in vivo* experiments, leads to faulty interpretation because of the presence of the thyroid gland which is the master organ for accumulation of iodide. Hence, an *in vitro* system is always desirable to overcome this problem. Iodide concentrating activity of mouse submaxillary gland was demonstrated earlier in the slice experiments *in vitro* (Fletcher *et al.*, 1956). The similarities and dissimilarities of the iodide transport system of the submaxillary gland with that of the thyroid gland has also been reported in tissue slices (Wolff and Maurey, 1961). An extensive work has been done on dispersed thyroid cells after Tong *et al.* (1962) described a continuous flow trypsinization method for isolation of thyroid cells capable of concentrating iodide with subsequent formation of thyroid hormones. We have recently developed a method for the isolation of single cell from mouse submaxillary gland and have observed that these cells are

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capable of concentrating iodide in *in vitro* experiments. Various properties of this transport system in these isolated cells are being reported in the present communication.

Materials and methods

Chemicals

Protease from *Streptomyces griseus* (Pronase, 90,000 PUK units/mg) was purchased from Serva Feinbiochemica, Heidelberg, Federal Republic of Germany. Mercaptomethylimidazole (MMI), thiouracil, thyroid stimulating hormone (TSH), thyroid hormones, sodium azide, ouabain and N-ethylmaleimide were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Na^{131}I was supplied by Bhabha Atomic Research Centre, Bombay. All other chemicals used were of analytical grade.

Preparation of cells and assay of iodide transport

Male Swiss mice (20–25 g) of the Institute inbred strain were used throughout the experiments. Submaxillary glands from 8 mice were finely minced, placed in a fluted conical flask containing 12 ml of Krebs-Ringer bicarbonate (KRB) buffer having 1·3 mM CaCl$_2$ and 3 ml of 100 mM glucose. The content was stirred for 1 h in the presence of 0·5 mg pronase. The supernatant solution was decanted through glasswool and centrifuged at 2000 *g* for 10 min to collect fraction 1 cells. Rest of the tissue was resuspended with the above medium and treated with 0·5 mg pronase for another 1 h to collect fraction 2 cells. Finally fraction 3 cells were collected similarly from the rest of the tissues after further treatment with 0·5 mg of pronase for 2 h. The adhering pronase in each cell fraction was washed out with 4 ml of KRB buffer and the cells of each fraction were finally suspended in 0·5 ml of the KRB buffer. The combined cell volume when estimated was found to be around 100 μl. At least 80% of the cells were viable as observed by exclusion of trypan blue. All these fractions were finally combined to a volume of 4 ml of KRB buffer before use. The assay system for the transport of iodide contained in a final volume of 1 ml of KRB medium: 0·5 ml of the cell suspension (cell volume 12 μl), 10 mM glucose, 10% mouse serum and 1 μM KI containing 5–10 μCi Na ^{131}I. It was incubated at 37°C for 2 h and the viability of the cells was checked again as before. No significant cell death was observed during this period of incubation. The cell suspension was centrifuged at 1000 *g* for 10 min, to separate the cells from the medium. The cell washing is not necessary as the amount of radioactivity trapped in the small volume of the cells used was found to be insignificant in comparison to the radioactivity inside the cells. Both the cells (C) and the medium (M) were counted separately in a gamma ray spectrometer and iodide transport (iodide concentration) was expressed as C/M ratio which is calculated from cpm per ml of cell divided by cpm per ml of the medium.

Results

With a view to getting a population of cells rich in iodide concentrating activity, each fraction of cells was first assayed for the ability to concentrate iodide as shown

in table 1. The results show that the cells in each fraction were equally active in iodide concentrating activity. Hence a pooled cell fraction was used to study the mechanism of iodide transport throughout the subsequent experiments. Figure 1 shows the time course study of the iodide concentration process. The transport of

Table 1. Iodide concentrating activity in different fractions of isolated cells.

	Iodide concentration (C/M ratio)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Fraction 1	5·4	5·0	7·2	5·9
Fraction 2	7·2	6·0	4·8	6·0
Fraction 3	6·6	5·0	9·6	7·0

The method of isolation of individual cell fraction and assay of C/M ratio has been described in text. Each experiment was carried out using different cell preparation.
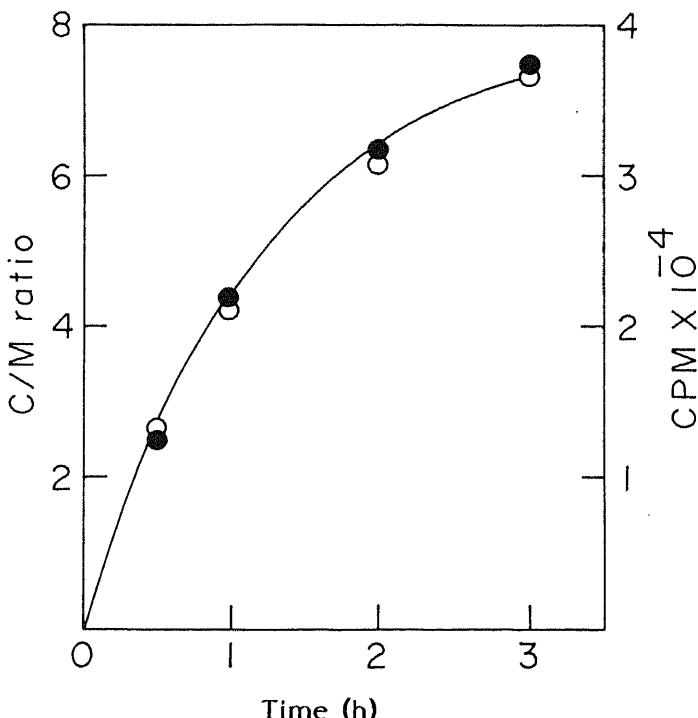
Figure 1. Kinetics of iodide transport. The cells were incubated with all the components at 37°C for different periods of time as indicated and C/M ratio (O) was calculated as described in the text; (●), indicates the radioactivity in the supernatant obtained after treating the cell homogenate with trichloroacetic acid.

iodide was almost linear for 2 h after which the rate decreased. The figure 1 shows that at 2 h, the concentrations of iodide inside the cells was 6 fold higher than that of the medium. The solid circle indicates the absolute amount of radioactivity present in the cytosol as recovered in the supernatant of the trichloroacetic acid treated cell homogenate. Chromatography and autoradiography of this supernatant indicates the presence of radioactivity with the iodide spot only. This transport of iodide was found to be linear when 0·2–2 mg of the cell protein was used in the assay system. Figure 2 indicates the effect of different concentrations of iodide in the medium on iodide concentrating activity. The activity was found to be linear up to a concentration of 0·5 μM KI above which the activity tends to saturate. The Lineweaver-Burk plot given as the inset of figure 2 shows that the transport system has the K_m of 0·3 μM for iodide with a V_{max} (C/M ratio) of 8·3. Table 2 demonstrates the effect of some inhibitors on the iodide transport activity. The cells usually exhibit a C/M ratio of 5–8. This activity is completely lost when the cells were heated at 100°C for 2 min (data not shown). Antithyroid drugs such as MMI and thiouracil strongly inhibit the iodide transport in these cells. Metabolic inhibitors such as azide and cyanide also inhibit the transport system. Ouabain, the inhibitor of Na^+–K^+ ATPase, causes only 30–40% inhibition. N-Ethylmaleimide, a sulphydryl reagent, has a slight stimulatory effect on the transport of iodide. Of the pseudohalides such as thiocyanate and perchlorate, only the former significantly
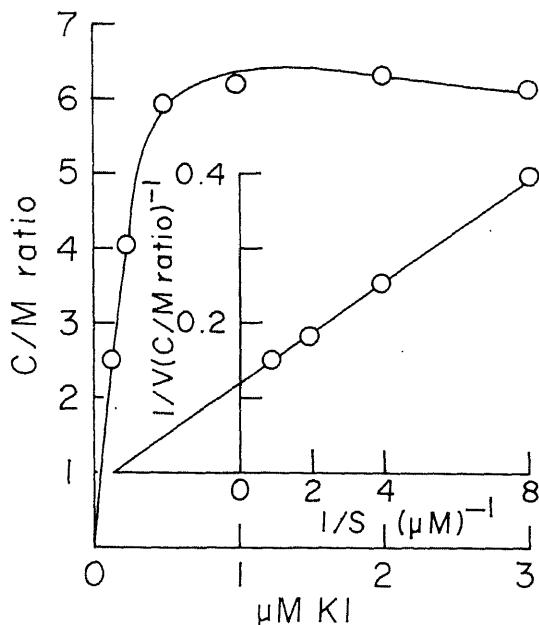
Figure 2. Effect of different concentrations of iodide on iodide transport. The cells were incubated with all the components containing different concentrations of KI in the presence of $Na^{131}I$ (keeping the specific radioactivity constant). The C/M ratio was calculated as described in the text. (C/M)$^{-1}$ was plotted against (μM KI)$^{-1}$ to determine the K_m as shown in the Lineweaver-Burk plot in the inset.

Table 2. Effect of some reagents on iodide concentrating activity

	Iodide concentration (C/M ratio)		
	Expt. 1	Expt. 2	Expt. 3
Control Cells	6·0	5·7	7·4
+MMI[a]	2·7	1·5	2·8
+Thiouracil[a]	2·7	1·9	2·5
+Azide[a]	2·9	1·9	1·5
+Cyanide[a]	2·6	2·0	3·0
+Ouabain[a]	4·0	4·0	5·0
+N-ethylmaleimide[a]	8·3	9·5	7·9
+Thiocyanate[a]	1·5	2·0	2·8
+Perchlorate[a]	4·1	5·0	7·9

[a] 1 MM.
The cells were preincubated with indicated concentrations of the reagents at 37°C for 10 min in the presence of all the components before the addition of serum, KI and [131]I. Three different cell preparations were used in the 3 experiments.

competes with the transport of iodide. It is known that iodide transport in the thyroid gland is stimulated by thyroid stimulating hormone. However, neither thyroid hormone nor the thyroid stimulating hormone has any significant effect on the iodide concentrating activity of the submaxillary cells as shown in table 3.

Table 3. Effect of thyroid hormones and thyroid stimulating hormone on iodide concentrating activity.

	Iodide concentration (C/M ratio)	
	Expt. 1	Expt. 2
Control cells	6·0	7·3
+Thyroxine 3 μg	5·2	7·0
+Triiodothyronine 3 μg	5·0	7·6
+Thyroid stimulating hormone 2 mu	6·0	9·6

The cells were preincubated at 37°C for 10 min in the presence or absence of the indicated amount of thyroid hormones or thyroid stimulating hormone before starting the transport with radioiodide. Expt. 1 and 2 represent similar experiments using two different preparation.

Discussion

Although iodide concentrating activity has been demonstrated *in vitro* in slices of mouse salivary gland (Fletcher *et al.*, 1956; Wolff and Maurey, 1961), this is perhaps the first report to demonstrate that the isolated cell preparation from mouse submaxillary gland can also concentrate iodide in *in vitro* experiments. The C/M ratio of 5–8 of the submaxillary cells is comparable to the value reported earlier in *in vivo* experiments (Banerjee and Datta, 1982) as well as in tissue slices *in vitro* (Wolff and Maurey, 1961). However, the lower value is not unusual in the isolated cells because of the damage of the cell membrane during isolation procedure.

One of the most important criterion for a transport system is the substrate saturability. The transport of iodide in the isolated submaxillary cells shows saturation above 1 μM KI having a K_m value of 0·3 μM. This value is at least 3 times less than the value reported earlier in slice experiments (Wolff and Maurey, 1961). The reason for this increased affinity in the dispersed cells is not clear. However, the possibility of the proteolytic modification of the transport system by pronase during cell preparation may not be excluded. The second important criterion is the sensitivity to some inhibitors and competitors. Iodide transport or concentration in any tissue is measured after blocking the iodide organification, if any, with antithyroid drugs such as mercaptomethylimidazole or thiouracil (Wolff and Maurey, 1961; Tong *et al.*, 1962). Previous workers have reported that mouse submaxillary gland does not have the ability to catalyze the organification of iodide to form protein-bound iodine (Fletcher *et al.*, 1956; Tong *et al.*, 1962). However, our studies indicate that dispersed cells from mouse submaxillary glands can catalyze protein-bound iodotyrosine formation with the help of the perioxidase present in this gland, although the amount of organification may be as little as 10% of the iodide concentrated (Banerjee *et al.*, 1985). Chromatography and autoradiography of the cell homogenate after incubation of the submaxillary cells with radioiodide have indicated that about 90% of the iodide concentrated remains as free iodide. Since a very small amount of iodide is metabolised in the organification by the endogenous peroxidase, we have not included antithyroid drugs such as MMI during transport studies similar to that reported earlier in the slice experiments (Wolff and Maurey, 1961). Furthermore, our *in vitro* studies indicate that iodide transport in the dispersed cells is strongly inhibited in the presence of these antithyroid drugs, an effect which limits the inclusion of antithyroid drugs during the assay of iodide transport in these cells. We cannot afford any explanation as to how these antithyroid drugs inhibit the transport in the isolated cells. In this connection, it may be mentioned that the cells also isolated either by trypsin or by collagenase treatment also showed similar inhibition with the antithyroid drugs indicating that the effect is not attributable to the use of pronase only. The inhibition of the transport with cyanide and azide is expected as they act metabolic inhibitors of the cells. It has been reported earlier that iodide transport in salivary gland slices is inhibited with ouabain which is a specific inhibitor of Na^+–K^+–ATPase (Wolff and Maurey, 1961). The authors concluded that iodide transport is dependent on K^+-concentration and ouabain perhaps inhibits the iodide transport by blocking K^+ entry inside the cell (Wolff and Maurey, 1961). The isolated submaxillary cells, however, show only 30–40% inhibition of the

transport of iodide with ouabain, which is lower than the inhibition reported in the slice experiment (Wolff and Maurey, 1961). This partial loss of ouabain sensitivity may be due to damage of the transport system during isolation of the cells by proteolytic treatment either by pronase or trypsin. However, sensitivity to ouabain indicates that the energy for uphill transport of iodide may be provided by the hydrolysis of ATP catalyzed by the $Na^+ - K^+$-ATPase. The reason for slight stimulation of iodide transport by N-ethylmaleimide is not clear. However, the possibility of the presence of any sulphydryl group containing inhibitory protein controlling the iodide transport in these cells cannot be excluded. One of the most important properties of the iodide transport system is the competition by pseudohalides such as thiocyanate and perchlorate. Iodide transport in the salivary gland slices has been shown to be strongly inhibited in the presence of thiocyanate or perchlorate (Fletcher *et al.*, 1956). Thiocyanate also acts as a strong inhibitor of iodide entry in the dispersed submaxillary cells and appears to be more effective. than perchlorate. It is known that iodide transport in the thyroid gland is stimulated by TSH and this effect has also been demonstrated in isolated thyroid cells (Knopp *et al.*, 1970). However, TSH has been reported to exert no effect on the iodide transport in the salivary gland slices (Wolff and Maurey, 1961). Our results also show that neither TSH nor thyroid hormones have any significant effect on the iodide concentrating activity indicating the absence of specific receptors for TSH in the cells. This is in agreement with the previous findings that iodide accumulation in the extrathyroidal tissues *in vivo* is insensitive to TSH (Taurog *et al.*, 1959; Banerjee and Datta, 1982). It thus appears from the results presented that the submaxillary cells contain iodide carrier which actively transport iodide inside the cells.

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Hydrogen peroxide formation and lipid peroxidation in rat uterus—effect of hormones and vitamin E

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Abstract. The effect of estradiol-17β and progesterone given separately as well as in combination on the rate of hydrogen peroxide formation and lipid peroxidation in the uteri of ovariectomized rats was studied. Estradiol in 3 μg dose per day per animal elicited maximum stimulatory response and progesterone (100 μg), on the other hand, was without any such effect. However, progesterone given along with estradiol completely prevented the effect due to the latter. In the same way, vitamin E, a well known antioxidant was found to be extremely effective in protecting the uterus from the highly peroxidative action of estradiol-17β.

Keywords. H_2O_2-formation; lipid peroxidation; free radicals; malonaldehyde-thiobarbituric reaction; estradiol-17β; progesterone; vitamin E (dl-α-tocopherol).

Introduction

The presence of the enzyme catalase (EC 1·11·1·6 H_2O_2 : H_2O_2 oxidoreductase) and its substrate hydrogen peroxide in the uterine tissue of rat and human has been reported (Dasgupta *et al.*, 1972; Bansal and Dasgupta, 1980; Johri and Dasgupta 1980a, b; Chakrabarti *et al.*, 1982). Formation of many organic hydroperoxides such as unsaturated fatty acid (UFA) hydroperoxides including hydrogen peroxide and finally the thiobarbituric reactive malonaldehyde like substances as a result of free radical chain process in animal tissues, is now well documented (Wills, 1966; Menzel, 1970; Slater, 1972; Oshino and Chance, 1973; Brunori and Rotilio, 1984). Mechanisms of lipid peroxidation have been studied in rat liver, spleen, heart and kidney (Wills, 1966) and in brain (Barber, 1963) both *in vitro* and *in vivo* (Slater, 1972).

A similar study in animal uterine tissue appeared pertinent from two points of interest: (i) this organ is an active site of protein biosynthesis and of estrogen metabolism, one of the products of which is hydrogen peroxide (Johri, 1978) and (ii) free radicals which set up a chain of biochemical reactions producing numerous highly reactive intermediates (Slater, 1972) may play a role in the process of fertilization and implantation.

Abbreviations used: UFA, Unsaturated fatty acid; MA, malonaldehyde; SDS, sodium dodecyl sulphate; TBA, thiobarbituric acid; IUD, intrauterine contraception devices.

In the present investigation an attempt has been made to determine the effect of estrogen and progesterone on the rate of hydrogen peroxide formation and the degree of lipid peroxidation in the rat uterine tissue.

Materials and methods

Adult virgin regularly cycling female albino rats (Wistar strain) (140–150 g) supplied by a local dealer were maintained under standard husbandry conditions at the Institute on a complete diet (developed here) and water *ad lib*, for a fortnight before any treatment. Bilateral overiectomy was performed aseptically under ether anaesthesia. After a rest period of 10 days following surgery the animals were injected intramuscularly with estradiol-17β and progesterone dissolved in groundnut oil once daily for 4 consecutive days and sacrificed 24 h after the last injection. Both the uteri of each animal were carefully dissected out, trimmed free from adhering fat and extraneous tissues, and freed from intraluminal fluid by opening out the uteri. After some preliminary mincing with a scalpel it was then homogenized in glass homogenizer with 0·05 M phosphate buffer (pH 7·4) solution such that 1 ml of the final homogenate corresponded to 100 mg of tissue (wet weight). The homogenates were centrifuged at 2700 g for 10 min and the supernatants were used for various analyses. All the steps commencing from dissection of the tissue till the preparation of the homogenates were performed in cold (4°C).

The animals were divided into the following groups: (i) Intact normal, having regular estrous cycle; (ii) ovariectomized control, receiving treatment with groundnut oil only; (iii) ovariectomized receiving estradiol only; (iv) ovariectomized, treated with progesterone only; (v) ovariectomized, treated simultaneously with both estradiol and progesterone; and (vi) ovariectomized, treated with vitamin E at the rate of 10 mg per 100 g of body weight from day 1 of post-operation till the end of estradiol administration. The dose of hormones used is given in tables 1 and 2. Six animals were taken in each group.

Estimation of protein

Protein concentration of the homogenates of uteri was estimated according to the method of Lowry *et al.* (1951).

Estimation of lipid peroxidation in terms of malonaldehyde (MA): Thiobarbituric acid reaction (Ohkawa *et al.*, 1979).

The reaction mixture contained 0·1 ml of tissue homogenate, 0·2 ml of 8·1% sodium dodecyl sulphate (SDS), 1·5 ml of 20% acetic acid and 1·5 ml of 0·8% aqueous solution of thiobarbituric acid (TBA). The pH of 20% acetic acid was adjusted with 1 N NaOH to 3·5. The mixture was finally made up to 4·0 ml with distilled water and heated at 95°C for 60 min on an oil bath. After cooling under tap water, 1·0 ml of distilled water and 5·0 ml of a mixture of n-butanol and pyridine (15:1 by vol) was added and the mixture was shaken vigorously on a vortex mixer. After centrifugation at 2200 g for 5 min the absorbance of the organic layer (upper layer) was measured immediately at 532 nm using appropriate controls in a Carry 17D model spectrophotometer.

Table 1. Effect of graded doses of estradiol-17β and progesterone on the rate of H_2O_2 formation and lipid peroxidation in the uterine tissue of ovariectomized rat.

Group	Dose of hormones (μg/day/rat)	H_2O_2 formed (nmol/100 mg protein/h)	MA accumulated (μmol/100 mg protein/h)
Intact control		166·06±7·48 (6)	3·13±0·69 (6)
Ovx control		52·10±8.27 (6)	0·980±0·36 (6)
Ovx+estradiol			
	2·00	109·01±10·98 (5)	1·66±0·192 (6)
	3·00	124·50±3·525 (6)	2·13±0·059 (6)
	4·00	105·36±4·74 (5)	1·04±0·088 (5)
Ovx+progesterone			
	25	52·37±5·672 (5)	0·981±0·027 (5)
	50	56·09±1·762 (6)	0·973±0·0244 (6)
	100	67·89±2·3613 (6)	0·956±0·0211 (6)
	200	85·70±3·6309 (6)	1·03±0·0292 (6)
	400	91±1·6134 (6)	1·013±0·0137 (6)
	500	88·83±1·723 (6)	1·072±0·0512 (6)

Results are expressed as mean±standard error of the mean. Figures in parentheses indicate the number of rats. Hormones were administered at the dose indicated once daily for 4 consecutive days. Control rats received vehicle only.

Table 2. Effect of combined treatment with estradiol (E_2) and progesterone (P) on the rate of H_2O_2 formation and lipid peroxidation in the uterine tissue of ovariectomized rat.

Dose of estradiol/ progesterone (μg/day/rat)	H_2O_2 formed (nmol/100 mg protein/h)	MA accumulated (μmol/100 mg protein/h)
Solvent only*	52·10±8·27 (6)	0·980±0·36 (6)
E_2 3 μg+P 100 μg	87·17±7·56 (5)	1·074±0·059 (5)
E_2 3 μg+P 500 μg	238·83±14·54 (5)	1·21±0·0315 (5)

Results are expressed as mean±standard error of the mean. Figures in parentheses indicate the number of rats. Estrogen and progesterone were administered once daily at the dose indicated for 4 consecutive days.
* The control group received groundnut oil only.

Estimation of H_2O_2 (Lichtenberg and Wellner, 1968)

The reaction mixture, in a final volume of 1 ml, contained 40 μmol of Tris-HCl (pH 7·5), 1 nmol of scopoletin, and 10 μg of peroxidase both in buffer. The solution was preincubated for 5 min at 37°C; then 0·1 ml of tissue homogenate was added and incubated for 15 min at 37°C with mild shaking in a Dubnoff shaker and the reaction was stopped by the addition of 4 ml of 0·1 M sodium borate (pH 10) and the mixture was then centrifuged at 2200 g for 5 min. The fluorescence of the clear supernatant was measured at 25°C with excitation monochromator set at 395 nm and the emission monochromator set at 470 nm in a Perkin Elmer Spectrofluorimeter (Model MDF–44B). The control contained all the reagents except peroxidase. Student's 't' test was applied for the calculation of significance.

Results

Table 1 illustrates that estradiol-17β does, indeed, stimulate the formation of H_2O_2 and of free radicals production, as indicated by the production of MA, in the ovariectomized rat uterus and that the dose of 3 μg per day is optimal ($P < 0·001$). However, the maximal effects obtained does not reach the levels of these two parameters observed in the intact normal group. Progesterone even at a dose as high as 100 μg per day is without any such effect on the uterus, although a massive dose (200–500 μg/day) appears to show some positive effect ($P < 0·05$).

Table 2 shows the results of the effect of treatment with an optimal dose of 3 μg of estradiol-17β along with progesterone in two different doses of 100 μg and 500 μg. It is found that the stimulatory effect of estradiol-17β on H_2O_2 production and lipid peroxidation has been substantially neutralized by 100 μg of progesterone. However, when a pharmacological dose of progesterone (500 μg) is used along with 3 μg of estradiol, a massive increase in the H_2O_2 formation is noted; however, no such rise in lipid peroxidation is found.

The results of administration of vitamin E on the increased formation of H_2O_2 and lipid peroxidation in ovariectomized rat uterus under the influence of estradiol-17β are presented in table 3. As expected, vitamin E prevented the

Table 3. Effect of vitamin E on H_2O_2 formation and lipid peroxidation in ovariectomized rat uterus.

Groups	H_2O_2 formed (nmol/100 mg protein/h)	MA accumulated (μmol/100 mg protein/h)
Ovx control	52·10±8·27*	0·980·36
	(6)	(6)
Ovx + estradiol	137·5±5·7	1·37±0·02
	(4)	(4)
Ovx + vitamin E + estradiol	91·7±6·0	0·48±0·17
	(4)	(4)

* Mean±SEM. Figures in parentheses indicate total number of animals. Estradiol dose 3 μg/day/animal for 4 days from day 10 post-operation; vitamin E 30 mg/day/100 g body weight of rat for 14 days post-operation.

increase in the formation of H_2O_2 as well as of lipid peroxidation which occurs under the influence of estradiol-17β. Furthermore, it is noteworthy that the protective action of vitamin E against free radical mediated lipid peroxidation is highly pronounced; the production of MA is reduced to half of that observed in the ovariectomized control uterus ($P < 0.001$).

Discussion

Dasgupta and coworkers (Bansal and Dasgupta, 1980; Johri and Dasgupta, 1980a, b; Chakrabarti *et al.*, 1982; Nayyar *et al.*, 1983) demonstrated an increase in the production of H_2O_2 in the uteri of rat and of human under the influence of intrauterine contraceptive devices (IUD) *in utero* and that such rise in the rat uterus was also dependent on the blood level of estrogen. It was suggested that H_2O_2 being a highly toxic metabolic intermediate could explain the contraceptive action of IUD's. Meanwhile, evidence accumulated suggesting that H_2O_2 is an active intermediate in the redox process initiating the production of free radicals in various tissues of the body, with a potential to cause tissue injury (Menzel, 1970; Slater, 1972; Janzen, 1980; Brunori and Rotilio, 1984). Experiments *in vivo* and *in vitro* have demonstrated the production of free radicals under the influence of poisonous substances like CCl_4 on the basis of the production of MA which produces a coloured substance with TBA, in various tissues, mainly in liver (Slater, 1972). However, no published report is known of similar studies in the uterine tissue which is a target for estradiol, and metabolism of which is reported to produce hydrogen peroxide (H_2O_2) (McNabb and Jellinck, 1976; Johri, 1978).

The results presented in table 1 clearly show that both H_2O_2 formation and lipid peroxidation occur in the rat uterus under normal physiological condition and both these parameters are drastically lowered ($P < 0.01$) following ovariectomy. However, treatment of spayed animals with estradiol-17β, but not with progesterone, restores substantially ($P < 0.01$) the preoperative levels in the uterus. Thus it is apparent that H_2O_2 formation and lipid peroxidation in the rat uterus are normal physiological events and are partly, but not entirely, estrogen dependent; possibly in intact normal animals other pathways besides metabolism of estrogen (McNabb and Jellinck, 1976; Johri, 1978) are operative in the uterus for the production of free radicals (Slater, 1972).

The effects of combined treatment with estradiol-17β and progesterone (table 2) are as expected to some extent. The well documented antiestrogen effect of progesterone has been clearly manifested and the stimulatory influence of estradiol on free radical formation has been practically neutralized ($P < 0.01$). However, it was interesting to note that a massive dose of 500 μg of progesterone along with estradiol-17β records a phenomenal increase in the level of H_2O_2 without affecting the extent of lipid peroxidation. Questions arise about the fate and disposal of such high quantities of H_2O_2. No explanation can be offered on the basis of the present data. However, it seems possible that the excess amount of progesterone may undergo degradation or is disposed of by a mechanism involving H_2O_2. *In vitro* studies may be useful in solving these questions. Many antioxidants, synthetic as well as natural, are known to effectively prevent lipid peroxidation in canned food materials (Slater, 1972). Vitamin E (α-tocopherol-dl) is of particular interest so far

as *in vivo* protection of the living tissue is concerned (Lucy, 1978; Fukuzawa *et al.*, 1981). For example, Hochstein and Ernster (1963) demonstrated the antioxidant action of vitamin E in many diverse conditions *in vitro*. Draper and Csallany (1969) have also reviewed these effects of vitamin E.

In the present study, vitamin E was administered before the commencement of hormone (estradiol) treatment of the spayed animals. It was interesting to observe that the vitamin offers protection against the peroxidative influence of estradiol-17β in the uterus (table 3). Whereas the level of H_2O_2 production was significantly kept under control ($P < 0.01$) the level of lipid peroxidation was markedly diminished compared to the spayed control level ($P < 0.001$). Such remarkable diminution of lipid peroxidation reaching half the level of spayed control may mean that the vitamin E defends not only against H_2O_2 mediated free radical formation, but also against those produced by other pathways.

Thus on the basis of present study, it appears that (i) lipid peroxidation is a normal physiological phenomenon of the rat uterus; (ii) it is dependent on estrogen, (iii) progesterone can neutralise the stimulatory effect of estradiol on lipid peroxidation, (iv) a non-hormonal antioxidant like vitamin E is extremely effective in protecting the tissue against lipid peroxidation and (v) estradiol metabolism and H_2O_2 formation constitute the major pathway for the production of free radicals in the uterus.

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Ontogeny of human fetal catalase, superoxide dismutase and lipid peroxidation: A comparative study

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Abstract. A comparative study on the activity profile of catalase and superoxide dismutase, the two scavenging enzymes, as well as the developmental profile of lipid peroxidation in the human fetal brain, liver and kidney have been done for gestation periods ranging from 12 weeks to 28 weeks and beyond. The activity of the scavenging enzymes increase gradually in all the tissues with the advancement of pregnancy. Brain is an exception in case of catalase where the activity remains more or less same throughout the developmental period except in the case of fetuses, 28 weeks and above where significant decrease in the catalase activity is observed. A high level of lipid peroxidation is observed during early stages of development which declines thereafter.

Keywords. Catalase; superoxide dismutase; lipid peroxidation; ontogeny; antioxidant system.

Introduction

Free radicals have long been postulated in biological processes such as ageing and in certain diseased states (Bondareff, 1964; Zemen and Dyken, 1969), but the types of molecular damage that might occur to the human fetuses during the different gestational periods have been difficult to ascertain due to unavailability of samples. It is well known that certain oxidative enzymes produce superoxide free radical (O_2^-) by a one-electron reduction of molecular oxygen, which can readily react with polyunsaturated fatty acids or their derived free radicals to cause lipid peroxidation, thereby causing irreversible damage to the membrane systems resulting in the death of the affected cells or disturbance to the physiological function of living cells. By decreasing the concentration of the reactants, superoxide dismutase and catalase act in tandem to prevent formation of more damaging species (Misra and Fridovich, 1971). The activity and different properties of catalase have been investigated by a large number of workers in various mammalian tissues including human (Kar and Pearson, 1979; Kaplan and Groves, 1972).

In the present investigation we have conducted a comparative study on the activity profile of catalase and superoxide dismutase as also the developmental pattern of lipid peroxidation in human fetal brain, liver and kidney at different stages of gestation.

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Materials and methods

The human fetuses were obtained from therapeutic abortions (hysterotomised) up to 20 weeks of pregnancy from different nursing homes and medical termination of pregnancy clinics in Calcutta. Fetuses above 20 weeks were collected as stillborn. Gestational age was calculated from the period of amenorrhea and by considering the fetal weight, fetal crown-to-rump length and crown-to-foot length. Beginning from the 12th week, the fetuses were grouped at 3 week intervals up to 27 weeks and one group included fetuses of 28 weeks and above. The bodies of the fetuses were stored in a freezer (−4°C for 1–9 h) immediately after death and the brain, liver and kidney tissues were removed usually within 1–3 h after death at a temperature of 0–4°C.

Tissues were homogenised in a Teflon homogeniser (0–4°C) in 0·15 M KCl to produce a 10% homogenate (w/v). The homogenates were centrifuged at 105,000 *g* for 60 min and the supernatant fractions were used as the enzyme source. The activity of catalase was estimated according to the method of Beers and Sizer (1952). Cu-Zn superoxide dismutase activity was assayed according to the method of Marklund and Marklund (1974). Total homogenates were used for assaying malondialdehyde formation according to the method of Utley *et al.* (1967). The protein content was estimated according to the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

The results indicate that the activity of catalase in human fetal brain does not show any significant change throughout the gestational period but a significant decrease in the activity is noted at the late gestational age, whereas in liver and kidney the enzyme activity increases in parallel with the advancement of pregnancy (table 1). In the case of superoxide dismutase, the activity increases in all the 3 tissues studied (table 2). High level of lipid peroxidation can be detected at early stages which decline thereafter with the advancement of pregnancy. Brain always shows highest level of lipid peroxidation in comparison with the other two tissues (table 3).

Table 1. Age related changes of catalase activity in human fetal brain, liver and kidney.

Gestational ages in weeks	Brain	Liver	Kidney
12–15 (6)	0·46±0·050	2·26±0·131	1·28±0·102
16–19 (6)	0·43±0·011	2·37±0·159	1·35±0·098
20–23 (5)	0·40±0·079	2·82±0·103[b]	1·74±0·073[b]
24–27 (5)	0·45±0·025	2·99±0·213	2·05±0·119[b]
28 and above (5)	0·27±0·020[b]	3·27±0·299	2·10±0·209[a]

For calculation of *P* value, the result of each group is compared with the result of the preceding group.
Specific activity is expressed as mol of H_2O_2 decomposed/mg protein/min × 10^{-2}. Values in the parentheses indicate number of cases studied. Results are expressed as mean±SD value of experiments carried out in each case.
[a] *P*<0·01 (significant). [b] *P*<0·001 (highly significant)

Table 2. Age related changes of superoxide dismutase activity in human fetal brain, liver and kidney.

Gestational ages in weeks	Brain	Liver	Kidney
12–15 (6)	1·74±0·093	1·82±0·077	0·94±0·085
16–19 (6)	1·97±0·112[a]	1·99±0·098[a]	1·87±0·104[b]
20–23 (5)	2·22±0·153[a]	2·15±0·089	2·02±0·196
24–27 (5)	2·35±0·114	2·24±0·092	2·54±0·191[a]
28 and above (5)	2·59±0·143	5·58±0·321[b]	3·92±0·219[b]

For calculation of *P* value, the result of each group is compared with the result of the preceding group.

Specific activity is expressed as unit/mg protein. One unit of superoxide dismutase is that amount of enzyme required to inhibit the autoxidation of pyrogallol by 50% in a standard system of 3 ml. Values in the parentheses indicate number of cases studied. Results are expressed as mean±SD value of experiments carried out in each case.

[a] *P*<0·01 (significant). [b] *P*<0·001 (highly significant).

Table 3. Age related changes of lipid peroxidation level in human fetal brain, liver and kidney.

Gestational ages in weeks	Brain	Liver	Kidney
12–15 (6)	10·41±0·594	4·40±0·403	2·48±0·108
16–19 (6)	7·89±0·227[b]	2·80±0·198[b]	1·98±0·119[b]
20–23 (5)	5·93±0·138[b]	1·81±0·094[b]	1·71±0·090[a]
24–27 (5)	5·65±0·249	1·32±0·109[b]	1·25±0·070[b]
28 and above (5)	3·30±0·166[b]	1·12±0·072[a] .	0·58±0·033[b]

For calculation of *P* value, the result of each group is compared with the result of the preceding group.

The value is expressed as nmol malondialdehyde formed/mg protein. Values in parentheses indicate number of cases studied. Results are expressed as mean±SD value of experiments carried out in each case.

[a] *P*<0·01 (significant). [b] *P*<0·001 (highly significant).

Discussion

Lipid peroxidation is primarily an outcome of the formation of free radicals, peroxides and superoxide anions, the detoxification of which are effected by glutathione peroxidase, superoxide dismutase and catalase (Basu *et al.*, 1983). The developing pattern of glutathione peroxidase activity has been reported elsewhere (De *et al.*, 1985). The fetal age-related increase in the activities of superoxide dismutase and catalase is in conformity with the observation made by Sekiba and Yoshioka (1979) and Nohl *et al.* (1979). It is probable that in the mid and late gestational phases when the cells are becoming more and more aerobic, these two enzymes are subjected to increased turnover in an attempt to overcome the deleterious effects of the toxic reactants. The low activity of catalase in brain throughout the gestational ages is of particular relevance to the low activity of catalase and haem-containing peroxidases in the central nervous system (Seiler, 1969).

It has also been reported that the early period of brain growth in human fetus is marked by an increase in hexose monophosphate shunt activity (Sengupta *et al.*, 1985) that generates reducing power (NADPH) which can detoxify oxidants (free radicals, *viz.* O_2^- radical) contributing to the age related decline in structure and function. Thus the system is acquiring more antioxidant properties with the advancement of pregnancy thereby protecting the tissues against oxidative damage.

We know that throughout ontogeny, the exposure of brain, liver and kidney tissues to a changing environment involves processes of enzymatic adaptation as suggested by Anokhim (1956) correlating with the gradual acquisition of adult functions and ultimately adult behaviour patterns. A probable clinical significance of the present investigation is that any deficiency of these two scavenging enzymes may lead to toxic effects in these rapidly proliferating tissues by the accumulation of hydrogen peroxide, which may in turn affect their normal growth and well-being.

Acknowledgements

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Purification and characterization of tubulin from the catfish *Heteropneustes fossilis*

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Abstract. Tubulin was purified from the brain of the catfish *Heteropneustes fossilis* by cycles of temperature-dependent assembly and disassembly. Fish tubulin assembles into microtubules in the absence of high molecular weight microtubule associated proteins. Its subunits comigrate with goat brain α and β tubulin subunits and is composed of 4 major α and β tubulins each as analyzed by isoelectric focusing and two dimensional gel electrophoresis. Peptide mapping showed it to be very similar to goat brain tubulin. Polymerization of catfish brain tubulin occurs optimally between 18–37°C and the critical protein concentrations of assembly at 18°C and 37°C are the same, as opposed to mammalian brain tubulins.

Keywords. Tubulin; *Heteropneustes fossilis*; purification and properties.

Introduction

Microtubules (MT) are present in all eukaryotic cells and they play important roles in cell division, morphogenesis, intercellular transport, secretion and motility (Dustin, 1978). Much of our present knowledge of MT structure and biochemistry results from studies of the *in vitro* assembly of brain MT, whose building block is the protein tubulin, a heterodimer consisting of an α chain and a β chain, each with a molecular weight (M_r) of about 55,000 (Roberts and Hyams, 1979; Biswas *et al.*, 1981). The use of peptide mapping (Little *et al.*, 1981), aminoacid sequence analysis (Ponstingl *et al.*, 1982) and recombinant DNA techniques (Cleveland and Kirschner, 1982; Neff *et al.*, 1983) has shown that α and β tubulins are conserved polypeptides which are products of non-identical tubulin genes.

Tubulin from brain of homoiothermic organisms such as chick and mammals has similar characteristics and requirements for polymerization. Such tubulin will spontaneously polymerize *in vitro* into microtubules at physiological temperatures, pH, ionic strength and in the presence of Mg^{2+} and GTP (Timasheff and Grisham, 1980; Biswas *et al.*, 1981; Correia and Williams, 1983). A set of proteins known as microtubule-associated proteins (MAPs), copurify with mammalian and chick brain tubulin. These proteins, which usually appear as high M_r bands on sodium

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Abbreviations used:. MT, microtubules; MAPs, microtubule-associated proteins; SDS, sodium dodecyl sulphate; PIPES, 1,4-piperazinediethane sulphonic acid; EGTA, ethylene glycol-bis (β-amino-ethylether)-N,N,N′,N′-tetraacetic acid; EDTA, ethylene diamine tetraacetic acid; M_r, molecular weight.

dodecyl sulphate (SDS) gels (Murphy and Borisy, 1975) and contain an active factor referred to as τ (Weingarten *et al.*, 1975) are required for either initiation or growth of microtubules.

Poikilothermic animals like fishes live in water where temperature goes much below the body temperature of homoiothermic animals. Hence, in contrast to homoiotherms, microtubules present in the tissues of poikilotherms must be assembled from tubulins at low temperature and they must remain assembled under those conditions. So far, study on tubulin from fishes has been scanty. Langford (1978) has shown that dog fish brain tubulin is more stable at low temperature and differs from mammalian tubulin in MAPs and effect of Ca^{2+}. As a part of our investigation on the developmental biology of catfish *Heteropneustes fossilis* (Sarkar *et al.*, 1979; Chaudhuri *et al.*, 1979), we are interested in studying the differential regulation of tubulin gene expression during embryonic development of this poikilotherm.

As described in this paper, the first step in our study was to purify and characterize catfish tubulin. This tubulin has been found to differ from mammalian tubulin in terms of MAPs, isoelectric points, temperature stability and assembly characteristics, but migration on SDS/polyacrylamide gels and peptide maps are very similar. When this work was completed, a report on tubulin with somewhat similar properties from antarctic fish appeared (Williams *et al.*, 1985).

Experimental

Materials

GTP, colchicine and 1,4-piperazinediethane sulphonic acid (PIPES) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. DEAE cellulose (DE52) paper was obtained from Whatman. Podophyllotoxin was from Aldrich Chemical Co., Milwaukee, Wisconsin, USA. *Staphylococcus aureus* V8 protease (Miles) was a gift from Dr S. Biswas of this department. Ampholines were obtained from LKB, Sweden. [^{3}H]-Colchicine was purchased from New England Nuclear Corporation. All other chemicals used were of analytical reagent grade.

Preparation of catfish brain tubulin

All the operations were carried out at 4°C unless otherwise stated. The brains of catfish were removed immediately after decapitation and kept in ice. The tissue was homogenized in cold homogenization buffer (100 mM PIPES/0·5 mM $MgCl_2$/1 mM ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)/0.1 mM GTP, pH 6·9, at a ratio of 1 ml/g tissue). The homogenized tissue was centrifuged at 100,000 g for 1 h in Beckman L5-50 ultracentrifuge. The resulting supernatant was purified through two cycles of assembly-disassembly in a buffer containing (100 mM PIPES, pH 6·9/1 mM $MgCl_2$/1 mM EGTA/1 mM GTP) according to the methods of Shelansky *et al.* (1973). Glycerol (4 M) was added during assembly. The second cycle purified microtubule pellet was stored at −70°C. Goat brain tubulin used for comparison was also prepared by the above method.

Polymerization assay for microtubule assembly

All microtubule assembly experiments were done in assembly buffer (0.1 M PIPES, pH 6·9/1 mM MgCl$_2$/1 mM EGTA) containing 4 M glycerol. Microtubule assembly was monitored turbidimetrically at 400 nm with a Shimadzu Double-Beam UV-210A recording spectrophotometer equipped with water circulating arrangement. Polymerization was initiated with the addition of 1 mM GTP.

The critical concentration (C_{cr}) of assembly during the second cycle of polymerization of assembly was evaluated by turbidimetry (Gaskin *et al.*, 1974). Samples at various initial concentrations were assembled to steady state (apparent equilibrium) and the change in absorbance at 400 nm was plotted *vs.* total protein concentration. The apparent critical concentration was estimated by extrapolation of the linear regression line to the abscissa to zero turbidity, *i.e.* no assembly.

Colchicine binding assay

The DEAE-cellulose filter disc method of Weisenberg *et al.* (1968) was used as modified by Williams and Wolff (1972). Protein was determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as standard.

Gel analysis of tubulin

Slab gel electrophoresis was performed in 7·5% polyacrylamide gels containing 0·1% SDS at constant voltage of 120, essentially as described by Laemmli (1970). Isoelectric focusing and two dimensional electrophoresis were carried out essentially as described by O'Farrell (1975), with some modification. The first dimension isoelectric focusing was done in tube gels (12×2·5 mm). A gradient of pH 4–6 was established in a 4% polyacrylamide gel support using a 4:1 mixture of ampholines of pH range 4/6 and 3/10. The gels were prefocused for 15 min at 200 V, 30 min at 300 V and 30 min at 400 V. The samples (in 9·5 M urea, 2% (w/v) NP40, 2% ampholine and 5% β-mercaptoethanol) were loaded onto the gel at the basic end and the focusing was performed at 300 V for 16 h and at 800 V for 1 h for sharpening the bands. After this, a little sample loading buffer with bromophenol blue was added to the gel tubes and the gels were run for 10 min to mark the basic end. On termination of the run the gels were stained and destained after Gozes and Littauer (1978). The gels for second dimension were run at 25 mA/gel in a 7·5% polyacrylamide SDS gel slabs (11×15×3 mm) according to Laemmli (1970).

Peptide mapping

Fish and goat tubulins were electrophoresed in a 7·5% SDS polyacrylamide gel (Laemmli, 1970). The α and β tubulin subunits were visualized by staining and destaining briefly. The bands were cut out from the gel, soaked for 30 min in 10 ml of 0·125 M Tris-HCl, pH 6·8, 0·1% SDS and 1 mM ethylene diamine tetraacetic acid (EDTA). The gel slices after equilibration were placed in the sample wells of a 15% SDS polyacrylamide gel. The slices were digested in the stacking gel with different concentrations of V8 protease from *S. aureus* according to the method of Cleveland *et al.* (1977a). Gels were stained in 0·1% Commassie blue, 50% methanol and 10% acetic acid and destained by washing with 5% methanol −7·5% acetic acid.

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ation of tubulin from fish brain and its binding to [³H]-colchicine

andard method of isolation of tubulin by alternate cycles of polymerization polymerization (Shelansky *et al.*, 1973) gave about 600 μg protein from 2·5 g ain. Goat brain tubulin was isolated similarly and the yield was comparable. e SDS gel, both goat and fish brain tubulins had identical mobility. When fish n was subjected to cycles of assembly/disassembly *in vitro*, the resulting natant contained α and β tubulins (>95% of the total protein) and trace ties of MAPs with M_r less than M_r of tubulin *i.e.* 55,000 after the first cycle of bly (figure 1, 1X), which disappears after subsequent assembly/disassembly
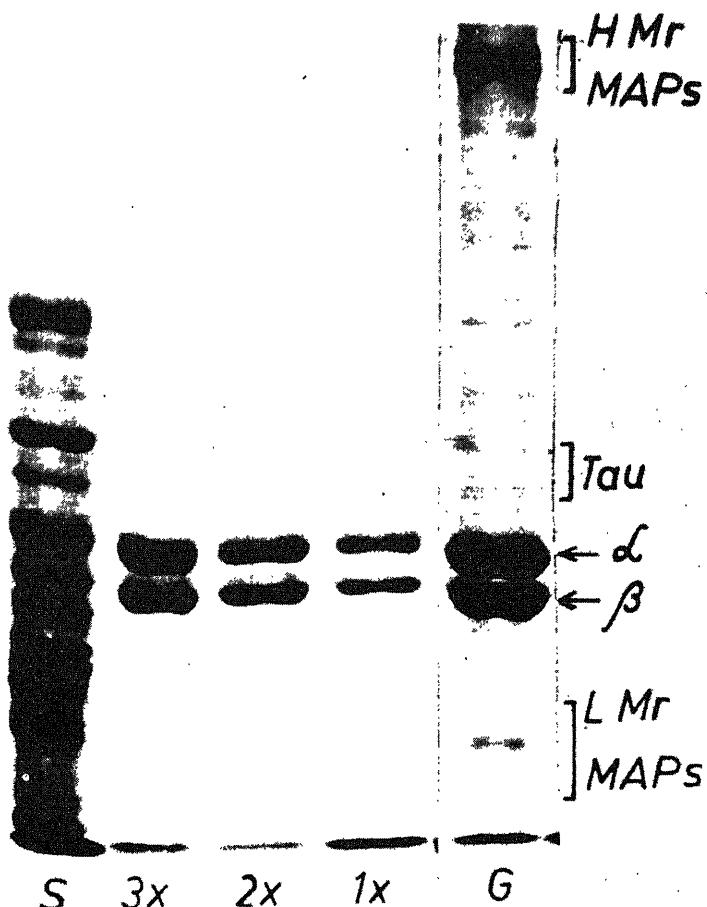
Figure 1. Purification of catfish brain tubulin. Catfish brain tubulin was purified by cycles of assembly and disassembly at 37°C and analyzed by 7·5% SDS-polyacrylamide gel and Coomassie blue staining, as described in 'experimental' section. (S), First cycle catfish brain supernatant; (1X), (2X), (3X), first, second and third cycle pellet of catfish brain tubulin. (G), second cycle goat brain tubulin.

(figure 1, 2X, 3X). No associated proteins corresponding to the γ protein of M_r 55,000–64,000 (Weingarten *et al.*, 1975; Cleveland *et al.*, 1977b), or to the high M_r MAPs (M_r 275,000–325,000) (Murphy and Borisy, 1975; Sloboda *et al.*, 1975) found in MT protein from vertebrate brain (as shown by goat brain tubulin here) have been observed in first cycle or second cycle polymerized preparations of catfish brain. The authenticity of fish tubulin was also tested by [^{3}H]-colchicine binding and it was found to bind with similar stoichiometry as in goat brain tubulin and competed by podophyllotoxin, another tubulin binding drug (table 1).

Table 1. Binding of [^{3}H]-colchicine to fish tubulin.

Sample	Protein amount μg	[^{3}H]-Colchicine bound cpm	Specific activity cpm $\times 10^{-3}$/ mg
Blank	—	360	—
Goat tubulin	125	8,000	6·4
Fish tubulin	125	6,000	4·8
Fish tubulin preincubated with podophyllotoxin	125	2,000	1·6

Binding of colchicine and competition by 1×10^{-6} M podophyllotoxin to goat and fish brain tubulin (incubation for 45 min at 37°C with 1×10^{-6} M [^{3}H]-colchicine).

Assembly of fish tubulin

Assembly of fish tubulin at physiological temperature: As the yield of tubulin after second cycle polymerization was not sufficient for many sets of experiment under the same condition, the assembly properties of fish tubulin after one cycle of polymerization and depolymerization were studied spectrophotometrically in presence of glycerol. Polymerization was initiated with addition of 1 mM GTP at 37°C. Polymerization appears to proceed without a lag and reaches a steady state value in 10 min (figure 2).

The assembly of mammalian brain tubulin has almost always been studied at 25–37°C. However, poikilothermic animals like fishes live in water where the temperature goes down well below the body temperature of homoiotherms and they must be capable of assembling and maintaining MT at these temperatures. Therefore, the ability of fish tubulin to assemble at 18–20°C was studied spectrophotometrically. Polymerization appears to proceed with slight lag and reaches steady state value in about 15 min (figure 2). When kept in ice, it depolymerizes in 10 min and shows fall in turbidity. The final turbidity neither increased nor decreased significantly at temperature above 20°C.

During isolation and purification also, both 20°C and 37°C were used for assembly and the total yields of tubulin protein from equal weights of brain tissue were similar at both temperatures. Also, the first cycle pellet of tubulin from fish brain is at least 95% pure at both the temperatures. Assembly also takes place in
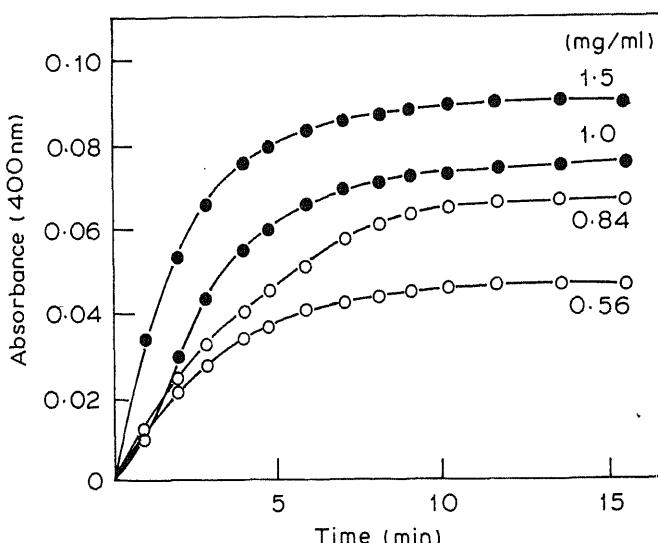
Figure 2. Assembly of catfish brain tubulin at 18°C and 37°C. Assembly in the second cycle of catfish brain tubulin was measured turbidimetrically in 100 mM PIPES/1 mM MgCl$_2$/1 mM EGTA/1 mM GTP and 4 M glycerol, pH 6·9 at 400 nm, at the indicated protein concentrations. (●), 37°C; (○), 18°C.

the absence of glycerol, but at a slower rate. When an aliquot of the sample was incubated in the absence of glycerol at 18°C or at 37°C for 30 min, MT protein pelleted down and analyzed on SDS gel, it showed α and β subunits indicating that the pellet indeed contained assembled microtubules.

Determination of critical concentration for assembly: The critical protein concentrations necessary for the assembly of fish brain tubulin at 18°C and 37°C were determined turbidimetrically by plotting the final steady state values of microtubule polymerization (A$_{400}$) against the total protein concentration of each sample. Extrapolation to zero turbidity yields an apparent critical concentration for fish tubulin below which no microtubule assembly takes place. At both temperatures, the apparent critical concentration of assembly of fish tubulin is almost the same *viz.* 0·26 mg/ml (range 0·26–0·28 mg/ml) (figure 3).

Characterization of fish tubulin

Gel analysis of fish tubulin: Upon isoelectric focusing, fish tubulin α and β each resolved into 4 major isoelectric variants in the pH range 5·4–5·8 (figure 4a). Isoelectric variants of goat tubulin, on the other hand were not as well resolved with this system (pH 5·2–5·5). The results show that fish and goat tubulin are composed of different isoelectric variants. On two dimensional gels also, the same fish tubulin isotypes can be seen. The purity of the tubulin preparation can be judged by the absence of other protein bands having the same isoelectric pH range (figure 4b).
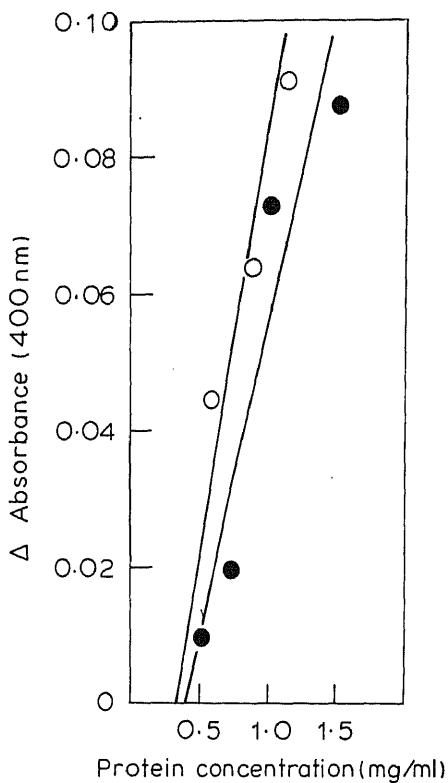
Figure 3. Turbidimetric determination of the critical concentration of assembly of fish brain tubulin. The experiment was done as in figure 2. Final steady state values at 18°C (O) and 37°C (●) were plotted against total protein concentration of each sample.

Peptide mapping: The proteolytic cleavage patterns of fish brain α and β tubulins were almost identical with those of goat brain α and β tubulins (figure 5). It seems fish tubulin is closely related to goat tubulin even though phylogenetically they are quite apart and one is a poikilothermic and other is a homoiothermic animal.

Discussion

The results indicate that fish tubulin self-assembles at low critical concentrations in the absence of known high M_r MAPs and even at low temperatures. Purified mammalian brain tubulin, in the same buffers as used in the present work, will only assemble at higher protein concentrations (1·4 mg/ml) at 37°C (Banerjee *et al.*, 1982; Himes *et al.*, 1977) and does not assemble at all below 18°C even in the presence of MAPs (Olmsted and Borisy, 1973). These assembly properties of fish tubulin similar to invertebrate poikilothermic animals like echinoderms and molluscs (Suprenant and Rebhun, 1981, 1984) suggest that they are conserved properties of tubulin of poikilotherms. Tubulin isolated from sea urchin outer doublet microtubule (Binder and Rosenbaum, 1978), dog fish brain extracts

Figure 4. Isoelectric focusing and two dimensional gel electrophoresis of fish brain tubulins. (a), Isoelectric focusing was performed as described in experimental section with 40 μg of fish and goat brain tubulin purified by two cycles of assembly and disassembly. (b), Electrophoresis in the second dimension of duplicate unstained IEF gel of catfish brain tubulin, obtained as in (a) was performed in 7·5% acrylamide-SDS gel. Staining was done in Coomassie blue.

(Langford, 1978), yeast (Kilmartin, 1981) or Ehrlich ascites cells (Doenges *et al.*, 1979) also self-assemble in the absence of known high M_r MAPs. The significance of low M_r proteins which appear in trace amounts after first cycle of assembly is not clear as they disappear after subsequent assembly cycles showing that they do not represent an absolutely necessary factor for assembly of fish brain tubulin. Low M_r proteins have been reported in oocytes of sea urchin and surf clam (Suprenant and Rebhun, 1981, 1984) and flagellar tubulin of sea urchin (Binder and Rosenbaum, 1978), some of which may be related to the low M_r MAPs (M_r 30,000–35,000) present in MT proteins isolated from bovine brain (Berkowitz *et al.*, 1977). Alternatively, they may represent MT binding, assembly promoting polypeptides similar to the fragments of MAP-2 (M_r 32,000–39,000), produced by proteolytic cleavage (Vallee, 1980). Further work is necessary to determine what role, if any, the low M_r proteins play in the regulation of fish tubulin assembly.

Figure 5. Proteolytic digestion patterns of α and β tubulin subunits from catfish and goat brain. Catfish and goat brain tubulin α and β subunits were separated on a 7·5% SDS-polyacrylamide gel and the cut out bands containing 10 μg protein were digested in a 5% stacking gel with 0·25 μg of *S. aureus* V8 protease and the resulting peptides were separated in a 15% SDS-polyacrylamide gel according to Cleveland *et al.* (1977). (a), (b) Catfish and goat brain α tubulins, and (c), (d) catfish and goat brain β tubulins, respectively.

The assembly of fish tubulin at low temperature shows it to be more stable than mammalian brain tubulin and the temperature of isolation (either 18–20°C or 37°C) does not significantly alter the yield. Similar temperature stability has been reported in case of dog fish brain tubulin (Langford, 1978), invertebrate poikilothermic animals like echinoderms and molluscs (Suprenant and Rebhun, 1981, 1984).

Isoelectric focusing and two dimensional gel electrophoresis resolves catfish tubulin into several isoforms (figure 4). These variants are different from goat brain tubulin isoforms. Microheterogeneity of tubulin from several organisms have been observed and is generally indicative of tubulin multigene families (Alexandraki and Ruderman, 1981; Mischke and Pardue, 1982; Hall *et al.*, 1983; Lopata *et al.*, 1983).

Post translational or post transcriptional modification may also generate micro-heterogeneity (Diez *et al.*, 1984). In the present case, it cannot be said whether the different tubulin isoforms are products of different genes. Studies on catfish tubulin gene families are now in progress to resolve this point.

The proteolytic-cleavage patterns of fish tubulin subunits after digestion with *S. aureus* protease were very similar to those of mammalian tubulin. This is not very surprising in view of the fact the peptide maps of *Artemia* tubulin subunits have been found to be very similar to bovine brain tubulin subunits in spite of the phylogenetic distance between these two organisms (Macrae and Luduena, 1984). Despite the evolutionarily conserved protein sequence of the tubulin molecules, the gross biochemical behaviour of fish tubulin *in vitro* is somewhat different from mammalian brain tubulin.

Acknowledgements

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Effect of supplementation with exogenous fatty acid on the biological properties of a fatty acid requiring auxotroph of *Salmonella typhimurium*

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Abstract. The effects of changes in fatty acid composition of the cell membrane on different biological functions of *Salmonella typhimurium* have been studied with the help of a temperature sensitive fatty acid auxotroph which cannot synthesise unsaturated fatty acids at high temperature. On being shifted to nonpermissive temperature the cells continue growing for another one and half to two generations. The rates of protein and DNA syntheses run parallel to the growth rate but the rate of RNA synthesis is reduced. Further, there is a gradual reduction in the rate of transport of exogenous uridine and thymidine into the soluble pool. The transport process can be restored by supplementing the growth medium with *cis*-unsaturated fatty acids but not *trans*-unsaturated ones although the growth of the cells is resumed by supplementation with either *cis* or *trans*-unsaturated fatty acids. However, supplementation with *trans*, *trans*-unsaturated fatty acids leads to only partial recovery of the transport process. The rate of oxygen uptake is also affected in cells grown in the presence of the *trans*-unsaturated fatty acids, elaidic acid and palmitelaidic acid. Analysis of cells grown under different fatty acid supplementation indicate that fatty acid composition of the cell membrane, especially the ratio of unsaturated to saturated fatty acids varies with temperature shift and supplementation of the growth media with fatty acids.

Keywords. Transport; fatty acid auxotroph; *cis*- and *trans*-unsaturated fatty acids.

Introduction

The lipid composition of the cell membrane plays an important role in regulating the functions of the cells. The role of membrane lipid in various membrane associated biological functions of the cell has become discernible from the extensive studies carried out in different laboratories (*c.f.* Silbert, 1975; Cronan, 1978). Investigations have been carried out mostly with *Escherichia coli* and *Saccharomyces cerevisiae* and attempts have been made to alter the lipid composition of the

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Abbreviations used: MM, Minimal medium; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; PE, phosphatidyl-ethanolamine; PS, phosphatidyl serine; PG, phosphatidyl glycerol; PEGA, polyethylene glycol adipate; CL, cardiolipin.

cell by supplementation, genetic manipulation, alteration of the growth condition, etc. Effect of supplementation with *cis*- and *trans*-unsaturated fatty acids has not been studied. In the present investigation a conditional fatty acid auxotroph of *Salmonella typhimurium* which cannot synthesise unsaturated fatty acids at high temperature, say 37°C, has been used. The effects of supplementation of the medium with *cis*- and *trans*-unsaturated fatty acids on a variety of biological functions of the cell membrane have been studied. The primary object was to correlate the fluidity of the cell membrane with its functions by comparing the effects of *cis*- and *trans*-unsaturated fatty acids.

Working with the unsaturated fatty acid auxotrophs of *E. coli* several workers (Silbert and Vagelos, 1967; Silbert *et al.*, 1968, 1973) have demonstrated directly that the fatty acid composition of the membrane can be altered by supplying various fatty acids in the growth medium. Therefore it is most likely that similar alteration will take place in the fatty acid auxotroph of *S. typhimurium*. To alter the fluidity of the cell membrane the fatty acids used in the study varied in their chain length, degree of unsaturation and steric configuration. The systemic names and other details of the fatty acids used are provided in table 1.

Table 1. Fatty acids used.

Common name	Systematic name	No. of carbon atoms	Formula
Palmitic	*n*-Hexadecanoic	16	$CH_3(CH_2)_{14}COOH$
Palmitoleic	*cis*-Δ^9-Hexadecanoic	16	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
Palmitelaidic	*trans*-Δ^9-Hexadecanoic	16	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
Stearic	*n*-Octadecanoic	18	$CH_3(CH_2)_{16}COOH$
Oleic	*cis*-Δ^9-Octadecanoic	18	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
Elaidic	*trans*-Δ^9-Octadecanoic	18	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
Linoleic	*cis*-Δ^9-Δ^{12}-Octadecadienoic	18	$CH_3(CH_2)_4(CH=CHCH_2)_2(CH_2)_6COOH$
Linolelaidic	*trans*-Δ^9-Δ^{12}-Octadecadienoic	18	$CH_3(CH_2)_4(CH=CHCH_2)_2(CH_2)_6COOH$

Materials and Methods

Chemicals

[3H]-L-Leucine (5200 mC/mmol), [3H]-thymidine (6500 mC/mmol) and [14C]-uridine (13·4 mC/mmol) were obtained from Bhabha Atomic Research Centre, Bombay. 2,5-Diphenyloxazole (PPO) and 1,4 di 2-(4-methyl-5-phenyloxazolyl) benzene (Dimethyl POPOP) were purchased from Amersham/Searle Corporation, USA. The fatty acids used in the study were from Serdary Research Laboratories, London, Ontario, Canada and obtained as generous gifts from Prof. B. D. Sanwal, Department of Biochemistry, University of Western Ontario, Canada. Other chemicals were also of analytical grade.

Bacterial strain

S. typhimurium fabB2, a fatty acid biosynthetic mutant, which cannot grow at higher temperature (37°C or higher) unless supplemented with unsaturated fatty acid and is probably deficient in β-ketoacyl acyl carrier protein synthetase I (Hong and Ames, 1971) used in this study was a gift from Prof. B. Ames, Department of Biochemistry, University of California, Berkeley, California, USA. The strain LT2 was originally obtained from Prof. M. Levine, Department of Human Genetics, Ann Arbor, Michigan, USA.

Growth media

Minimal medium: *S. typhimurium* was normally grown in minimal medium (MM), the composition of which was as described by Chakravorty (1970). Glycerol (0·2%) was used as the carbon source.

Growth of fabB2: When the auxotroph was grown in media supplemented with different fatty acids, 0·04% Brij-58 was added to the MM. At this concentration Brij-58 had no effect on the growth rate or the overall growth extent of the bacterium. Fatty acids were added at a concentration of 0·4 mM. Growth was measured by following the turbidity of the cell suspension at 610 nm. An absorbance of 0·1 is equivalent to $1·3 \times 10^8$ cells per ml.

Measurement of oxygen uptake by the cells

Oxygen uptake measurement was based on the measurement of change in the quantity of dissolved oxygen in solution. The model 55 Oxygen Monitor of Yellow Spring Instrument Co., Yellow Springs, Ohio, USA was used for the purpose.

Isolation of lipids

Total lipid was extracted following the method of Bligh and Dyer (1959) using the solvent system chloroform : methanol (2 : 1 v/v), chloroform : methanol (1 : 2 v/v) and chloroform : methanol : water (1 : 2 : 0·8 v/v/v). The amount of extractant used in each case was 5 ml/200 mg of dry cell. This procedure was repeated 3 times for complete extraction of lipids. 2,6-Ditertiary butyl-*p*-cresol (50 mg/l) was added in the solvents to protect the unsaturated fatty acids from aerial oxidation.

Thin-layer chromatography (TLC) of lipids

The individual phospholipids were characterized by comparing the R_f values with authentic standards and by spraying with specific reagents, *e.g.*, ninhydrin for phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS); and molybdenum blue for general phospholipid. Silica gel G, made by Centron India, was used for the separation of phospholipid.

Estimation of phospholipids

Total phospholipid and individual phospholipids were estimated colorimetrically (Ames and Dubin, 1960). Polar lipids chromatographing at the origin of the TLC plates after separation of the neutral lipids with acetone were extracted thrice with

chloroform : methanol : water (65 : 25 : 4 v/v/v). The extracts were combined, concentrated *in vacuo*, and then separated by preparative TLC (plate size 20 × 14 cm, thickness 0·25 mm) using the solvent system chloroform : methanol : water (1 : 2 : 0·8 v/v/v). Separated bands were scrapped off, extracted with the above solvent system and quantitated by phosphorous estimation.

Esterification of lipids

Total lipid was subjected to hydrolysis in the presence of 0·1 M methanolic sodium hydroxide at room temperature for 24 h. After hydrolysis, it was neutralised with 0·1 M acetic acid and fatty acid was extracted with diethyl ether and esterified with diazomethane. Formation of methyl ester was checked by TLC using n-hexane: diethyl ether : acetic acid (80 : 20 : 1 v/v/v) as the developing solvent and by spraying with 2′, 7′ dichlorofluorescein (0·2% in ethanol).

Gas-liquid chromatography (GLC)

Analytical gas chromatograph (Pye Unicam series 104 chromatograph) fitted with dual flame ionization detector was used. The fatty acid methyl esters were analysed on 10% polyethylene glycol adipate (PEGA) column (1·8 m × 2 mm), coated on a solid support (diatomite CAW) of 100–200 mesh. Nitrogen at a flow rate 40 ml/min was used as the carrier gas. The operating temperature was kept at 180°C. Peaks of the fatty acid methyl esters were identified by (i) comparing retention times with those of reference methyl esters and (ii) determining the carbon number of component esters (Ackman, 1963). Quantitation of the peaks was done by the triangular technique.

Incorporation of [^{14}C]-uridine and [^{3}H]-thymidine into soluble pool and macromolecules

Growing cells were labelled for 1 min with [^{14}C]-uridine (3·7 nmol, 5×10^5 counts/min/ml) or [^{3}H]-thymidine (4 nmol, $4·4 \times 10^6$ counts/min/ml) as described earlier (Rao *et al.*, 1972). The extent of incorporation into the soluble pool was calculated by substracting the amount incorporated into acid-precipitable fraction from the total incorporation.

Results

Effect of fatty acid on the growth of mutant

The conditional auxotroph fabB2 of *S. typhimurium* can grow at nonpermissive temperature in MM if supplemented with unsaturated fatty acid. The generation times of the mutant in MM supplemented with different fatty acids were practically the same (table 2). The growth rate was slightly slower with *trans*-unsaturated fatty acids than with the corresponding *cis*-unsaturated ones except linolelaidic acid. The saturated fatty acids, however, failed to sustain the growth of the mutant at nonpermissive temperature. When shifted from 30°C (permissive temperature) to 37°C (nonpermissive temperature) the mutant grew for one and a half to two

Table 2. Generation time of fabB2 at 37°C in MM supplemented with different fatty acids.

Supplementation	Generation time (min)
Oleic	80
Elaidic	96
Palmitoleic	75
Palmitelaidic	90
Linoleic	85
Linolelaidic	92

The cells did not grow at 37°C without supplementation. The generation time of the cells at 30°C (without supplementation) is around 130 min.

generations and then lysed. When the cells were provided with elaidic acid they could only grow for 10–12 h after which they started lysing.

Effect of fatty acid supplementation on lipid composition

Total lipid as well as fatty acid composition of the cells grown under different experimental conditions were analysed to find out the respective changes. Results presented in table 3 indicate that phospholipid content is very low in cells grown in minimal media at 30°C. It varied a great deal in the cells grown at 37°C in the presence or absence of different fatty acids. However, there is no qualitative variation in phospholipid spectrum except for some quantitative differences. Phosphatidyl serine content of the cells grown in the absence of any supplementation either at 30°C or at 37°C for 3 h following a shift from 30°C is much higher than that of the cells grown at 37°C in the presence of fatty acids (table 3). The fact that cells grown at 30°C do not lyse but contain as much phosphatidyl serine suggests that phosphatidyl serine is not derived from the action of endogenous phospholipase released upon lysis of a fraction of cells. The PE constitutes the major component. The other components are CL, PG and PS. No other phospholipid could be detected. The ratio of acidic lipids (CL, PG and PS) *versus* Zwitterionic lipid (PE) varied a great deal.

The fatty acid profile (table 4) shows that the ratio of unsaturated fatty acid : saturated fatty acid in the cell membrane was more in *cis*-unsaturated fatty acid supplemented cells except in the case of linolelaidic acid, which is a *trans*, *trans*-unsaturated fatty acid and resembles *cis*-unsaturated fatty acids in physical properties. The ratio was very much reduced when the cells were grown in the presence of elaidic acid or at 37°C (for a few generations) which is the nonpermissive temperature for the synthesis of unsaturated fatty acids.

Rate of protein, RNA and DNA synthesis in mutant after temperature shift

The rate of protein synthesis after shift to nonpermissive temperature was found to be almost parallel to the growth rate upto 2 h, the period during which the cells

Table 3. Percentage of total lipid, total phospholipid and individual phospholipid of fabB2 mutant grown under different conditions.

		Growth condition						
	30°C	30°C and then shifted to 37°C for 3 h	Oleic acid at 37°C	Elaidic acid at 37°C	Palmito-leic acid at 37°C	Palmite-laidic acid at 37°C	Linoleic acid at 37°C	Linolela-idic acid at 37°C
Total lipid[†]	11·8	9·0	7·5	10·4	9·8	10·2	8·7	10·8
Phospholipid[*]	17·8	63·2	62·0	62·5	73·5	45·4	86·1	56·4
PE[**]	41·2	67·8	75·1	81·5	83·1	68·5	81·3	62·0
CL	11·2	4·7	15·3	6·2	13·0	17·6	10·2	22·6
PG	29·2	15·0	8·2	12·5	2·5	11·4	7·7	11·0
PS	12·4	12·5	1·4	0·2	1·4	2·5	0·8	4·4
Acyl-PG	—	—	—	—	—	—	—	—
$\dfrac{CL+\,PG+PS}{PE}$	1·12	0·47	0·33	0·23	0·20	0·45	0·23	0·61

[†] Percentage of dry weight of cell.
[*] Percentage of total lipid.
[**] Percentage of total phospholipid.
— Not detected.

Table 4. Total lipid fatty acid composition (wt. %) of fabB2 mutant grown under different conditions.

Fatty acid components		Growth conditions						
	30°C	30°C then transferred to 37°C for 3 h	Oleic acid at 37°C	Elaidic acid at 37°C	Palmito-leic acid at 37°C	Palmite-laidic acid at 37°C	Linoleic acid at 37°C	Linolelai-dic acid at 37°C
12:0	1·7	T	0·5	T	3·3	1·1	3·6	1·4
14:0	4·7	41·7	7·7	28·9	6·8	4·6	9·0	14·2
14:1	—	—	0·2	—	T	—	3·1	5·0
15:0	2·6	—	—	1·5	—	1·7	—	—
16:0	31·5	34·1	30·0	17·6	11·5	36·9	31·8	24·1
16:1	28·0	21·4	10·6	11·1	58·7	44·8	3·6	T
17:0	10·5	—	—	T	—	6·5	10·3	4·3
17:1	7·7	—	—	—	8·1	—	—	T
18:0	T	T	T	28·0	—	T	7·6	T
18:1	13·1	2·7	42·0	12·1	11·5	4·2	10·1	T
18:2	T	T	—	T	T	T	20·8	46·7
18:3	—	—	—	—	T	T	T	4·2
$\dfrac{\text{Unsaturated}}{\text{Saturated}}$ fatty acids	0·96	0·32	1·9	0·32	3·7	0·96	0·71	1·28

T, Trace; —, not detected.
The values represent average of 3 determinations.

grew exponentially (figure 1A). After this the rate starts declining (figure 1A). The rate of RNA synthesis is slightly reduced immediately after the shift and continues at a reduced rate (figure 1B). However, the incorporation of exogenous uridine into free pool is drastically reduced from the time of shift to the higher temperature and there is negligible incorporation after 1 h. A similar effect on the transport of extracellular thymidine into intracellular pool was observed (figure 1C), although the rate of DNA synthesis increased exponentially along with the exponential growth of cells.

Effect of supplementation with cis or trans isomers of different fatty acids

It was of interest to study whether the rate of transport at 37°C can be recovered on addition of unsaturated fatty acids into the medium. The fatty acids were added 1 h after temperature shift and the rates of incorporation of uridine into RNA and intracellular pool were followed. Three sets of *cis* and *trans*-isomers of fatty acids used in the experiment were palmitoleic-palmitelaidic, oleic-elaidic, and linoleic-linolelaidic. As shown in figure 1A the rate of RNA synthesis decreased following temperature shift but on supplementation with palmitoleic or palmitelaidic acid the rate of RNA synthesis recovered (figure 2A). The recovery was immediate in the case of palmitoleic acid supplementation whereas there was slight delay in the case of palmitelaidic acid. Such delay was consistent and not due to experimental errors. The effect of such supplementation on the rate of uridine incorporation into soluble pool, which reflects transport process, was however, somewhat different (figure 2B). Supplementation with palmitoleic acid resulted in the recovery of the rate of transport of uridine into cellular pool but palmitelaidic acid had no effect. Similar results were obtained with oleic and elaidic acid, the *cis* and *trans* isomers of mono-unsaturated fatty acid containing 18 carbon atoms (figure 2C and D).

When the supplementation was made with the double *trans*, *trans*-unsaturated fatty acid, linolelaidic acid, its effect on uridine uptake is strikingly different from that caused by palmitelaidic and elaidic acid. The most interesting observation was, however, that in the presence of the double *trans*-unsaturated fatty acid, the rate of uridine transport starts recovering following the addition of linolelaidic acid (figure 2E and F). The rate of transport is however, greater in the case of linoleic acid, *cis*-Δ^9, Δ^{12}-octadecadienoeic acid.

Relative rates of uridine incorporation

To confirm that the rate of uridine transport is regulated or influenced by the fatty acid composition of the membrane, further experiments were done with the cells grown in the presence of different fatty acids for a number of generations, the rationale behind the experiment being that if the cells are grown for several generations in medium containing a particular fatty acid their membrane lipids will contain predominantly that particular fatty acid. The two pairs of *cis*- and *trans*-unsaturated acids chosen for the experiment were oleic-elaidic and linoleic-linolelaidic. Cells were grown at 37°C for a number of generations in MM containing different fatty acids and were quickly washed free of adhering fatty acid in the medium by filtering and washing through Millipore filter with the same medium devoid of fatty acid. Cells were finally suspended in MM without any fatty
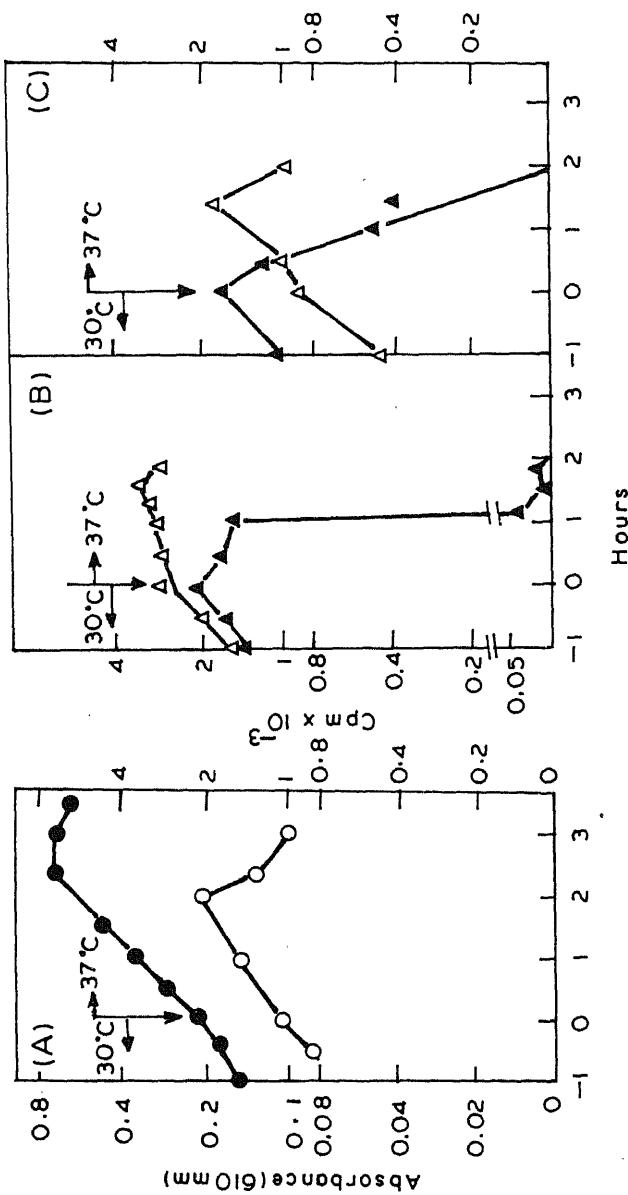
Figure 1. Synthesis of protein, RNA and DNA in the mutant fabB2 at nonpermissive temperature.

Cells (2·6×10⁸/ml) growing exponentially at 30°C in MM were shifted to 37°C at 0 h and the rates of growth and protein synthesis (A), incorporation of [¹⁴C]-uridine into RNA and intracellular pool; (B), incorporation of [³H]-thymidine into DNA and intracellular pool; (C), were measured as described under 'materials and methods'. (●), Growth; (○), rate of protein synthesis; (▲), incorporation of uridine (B) and thymidine (C) into intracellular pool.
(△), rate of synthesis of RNA (B) and DNA (C); (▲), incorporation of uridine (B) and thymidine (C) into intracellular pool.

Hours
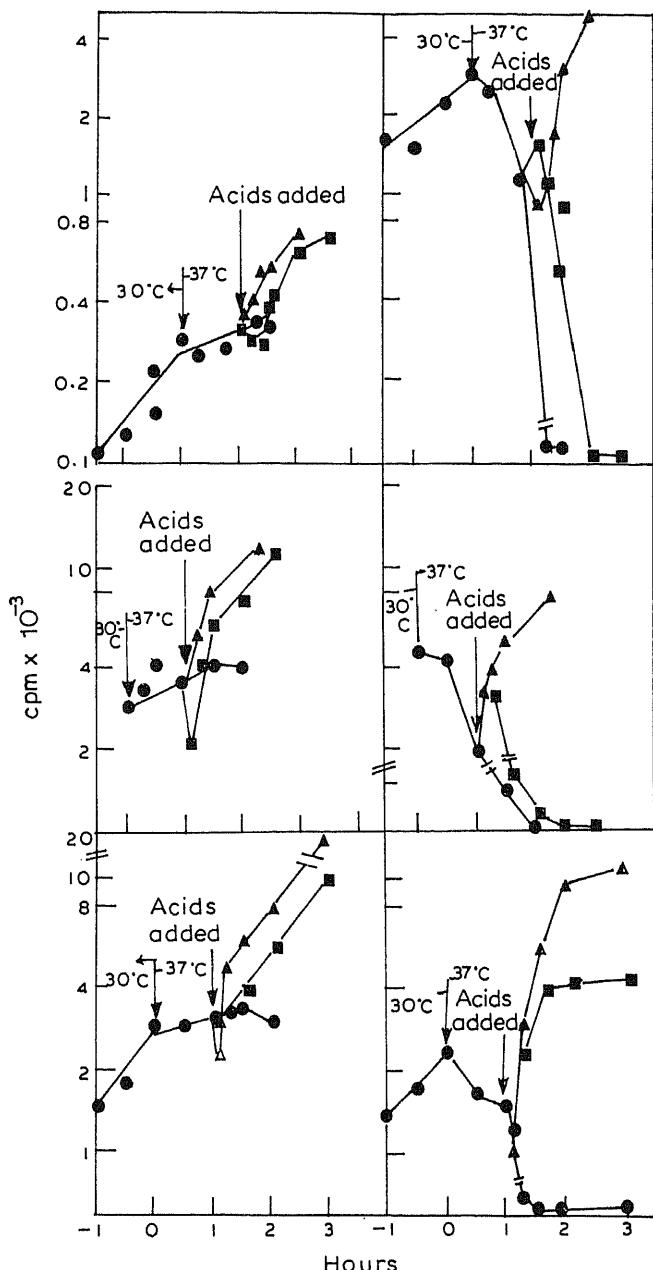
Figure 2. Effect of supplementation with *cis*- or *trans*-unsaturated fatty acids on the rates of incorporation of exogenous uridine into RNA and intracellular pool of fabB2 at nonpermissive temperature.

Cells (2.6×10^8/ml) growing exponentially in MM were shifted at 0 h from 30°C to 37°C as indicated in the figure. 1 h after the temperature shift they were divided into 3 batches. One batch was kept as unsupplemented control and to the remaining two batches two different fatty acids of the same chain length *cis*- or *trans-isomer* (0.4 mM) were added as follows: palmitoleic (A) and palmitelaidic acid (B); oleic (C) and elaidic acid (D) linoleic (E) and linolelaidic acid (F). The rates of incorporation of uridine into RNA and intracellular pool were estimated as described under 'materials and methods'. (●), Unsupplemented cells; (▲), supplemented with *cis*-unsaturated fatty acid; (■), supplemented with *trans*-unsaturated fatty acid.

acid. The incorporation of exogenous uridine into the RNA and soluble pool by such cells was then followed. The kinetics of incorporation presented in figure 3 agreed with the results presented in figure 2. The drastic reduction in the rate of incorporation of uridine into free pool was not the consequence of reduction in the rate of RNA synthesis (figure 3A) as cells treated with rifampicin (to block RNA synthesis) showed no reduction in the rate of incorporation of uridine into soluble pool (data not presented). There was no significant difference in the rates of uridine incorporation into RNA by linoleic and linolelaidic acid grown cells but there was difference both in the rates and extents of incorporation of uridine into free pool with this isomeric pair. These results confirmed our previous observation (figure 2 D and F) that the uridine transport could be recovered in the presence of linolelaidic acid, a *trans*, *trans*-unsaturated fatty acid.
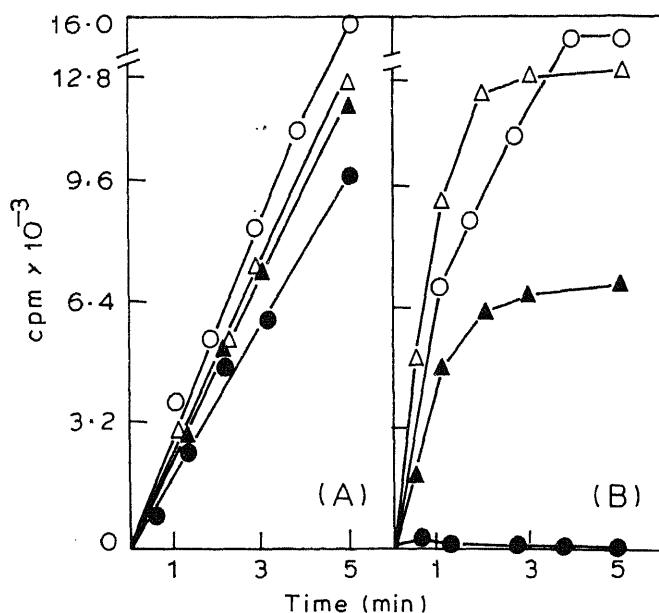
Figure 3. Incorporation of exogenous uridine into macromolecules (A) and soluble pool (B) of fabB2 grown in presence of *cis*- or *trans*-isomers of a mono- or di-unsaturated fatty acid.

The cells were grown for a number of generations and in media supplemented with different unsaturated fatty acid as indicated below. These exponentially growing cells were filtered through a Millipore filter, washed with media free of fatty acid (but containing Brij-58) and suspended in MM containing glycerol and no fatty acid at a density of $2·6 \times 10^8$ cells/ml. [^{14}C]-uridine (20 nmol containing 6×10^5 cpm) was added per ml of cell suspension. At desired times 1 ml aliquots of cell suspension were pipetted out for measuring the incorporation of radio-activity into macromolecules (A) and soluble pool (B) as described under 'materials and methods'. (O), Oleic acid; (●), elaidic acid; (△), linoleic acid; (▲), linolelaidic acid.

Effect of fatty acid supplementation on the cellular respiration

As the respiratory enzymes are mostly membrane-bound the rate of respiration of the cells grown under supplementation with different fatty acids at 37°C was followed (table 5). The rate of respiration of the cells grown in the presence of elaidic acid is considerably reduced. The mutant grown in the presence of palmitelaidic acid also respired at a reduced rate, but there is little effect on the rate of respiration of the linoleic and linoelaidic acid supplemented cells. This is in agreement with the results presented in figure 2. Thus it is evident that the rate of respiration like uridine transport also depends on the fatty acid composition of the membrane.

Table 5. Oxygen uptake on glycerol by fabB2 grown in media supplemented with different fatty acids.

Cells grown with	μl O_2 uptake/5 min/ $2 \cdot 6 \times 10^8$ cells
Palmitoleic	8·0
Palmitelaidic	5·5
Oleic	9·5
Elaidic	4·5
Linoleic	8·0
Linolelaidic	7·5

The cells were grown at 37°C for several generations in MM containing different fatty acids as listed above. The exponentially growing cells were harvested at room temperature, washed and suspended in MM containing glycerol as the carbon source without any fatty acid supplementation. The rate of respiration of these cells in such unsupplemented media was followed at 37°C. The values were corrected for endogenous rate which was negligible.
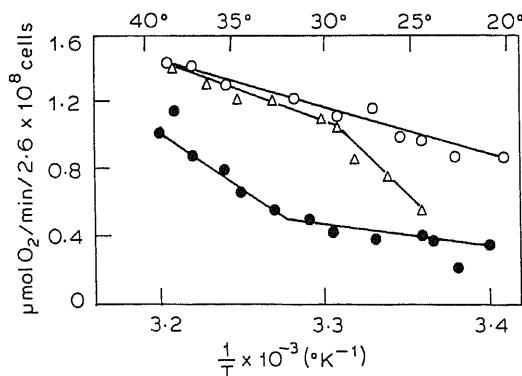
Figure 4. Effect of temperature on the rate of oxygen uptake by cells grown in presence of oleic, elaidic and palmitelaidic acids.

The rate of respiration was measured at different temperatures and plotted against the reciprocal of temperature in Kelvin Unit. The remaining conditions are described in the text. (O), Oleic acid; (●), elaidic acid; (△), palmitelaidic acid.

To ascertain that the fatty acid supplementation in the medium not only changed the fatty acid composition of the membrane but also affected its fluidity, the effect of temperature on the rate of respiration was followed. For this purpose the cells grown on oleic or elaidic or palmitelaidic acid were used. The cells were grown at 37°C for several generations in MM containing any of the above mentioned fatty acids. The exponentially growing cells were harvested, washed and suspended in MM containing glycerol as the carbon source (without any fatty acid supplementation). The rate of respiration was followed at different temperatures. The results presented in figure 4 shows the transition in the rates of respiration in the case of elaidic and palmitelaidic acid supplemented cells, the transition temperature being 32°C and 29°C, respectively. The oleic acid grown cells do not show such transition within the temperature range of the experiment (20°C–40°C).

Discussion

That the fatty acid composition of the cell membrane can be influenced by environment such as temperature and nutrition is well documented in the case of fatty acid auxotroph of *E. coli* (Cronan, 1978; Silbert and Vagelos, 1967; Silbert *et al.*, 1968, 1973; Sinosky, 1971), Yeast (Stokes, 1971), *Neurospora* (Martin *et al.*, 1981; Aaronson *et al.*, 1982) and some other organisms (Quinn, 1981). The results presented in this paper indicate that this is true in the case of the fatty acid auxotroph of *S. typhimurium*. Wild type *S. typhimurium* generally contains 90% phospholipid of the total lipid content (Ames, 1968). Variations to some extent have been observed in the fatty acid mutants of some organisms. However, 4 fold reduction in the phospholipid content of fabB2 mutant grown in MM at 30°C as compared to wild type (Ames, 1968) is rather significant. Decrease in the phospholipid content has been observed when *S. typhimurium* is grown in low phosphate medium (Cronan and Vagelos, 1972). A 10 fold decrease in phosphate resulted in 0·65 fold decrease in phospholipid content and a significant variation in the content of phospholipid. Results presented in table 3 indicate considerable variation in the phospholipid content of the cells grown under different conditions. The most striking feature of such alteration is the fact that the cells supplemented with *trans*-unsaturated fatty acid have a much lower ratio of unsaturated : saturated fatty acid. This suggests that the cells cannot either transport *trans*-unsaturated fatty acids into the cellular pool or incorporate into phospholipid as efficiently as *cis*-unsaturated one. Such distribution of saturated and unsaturated fatty acids must be affecting the fluidity of the membrane. The supporting evidence comes from the results presented in figure 4. The rate of respiration of cells grown in different fatty acids shows a characteristic transition temperature which is higher in elaidic acid grown cells than in oleic acid grown one. A more direct evidence involving measurement of the fluidity of the membrane vesicles prepared from cells grown under different conditions using electron spin labelled fatty acids or fluorescent probe is yet to be provided.

A number of parameters such as growth, RNA and DNA synthesis, transport of uridine and thymidine and respiration have been followed to study the biological functions of the cells. Of all the above parameters uridine transport has been studied in detail. On temperature shift for a period of 3 h, the unsaturated fatty

acid content of the cells decreased to 24%. *E. coli* auxotrophs starved for unsaturated fatty acids can grow well with 24% unsaturated fatty acid (Cronan and Gelman, 1973). However, fabB mutant of *S. typhimurium* lyses when the unsaturated fatty acid content is reduced to 24%. The type of unsaturated fatty acids present in the membrane of *E. coli* and *S. typhimurium* auxotrophs under the above conditions may be different and the physical properties of the membrane not only depend on the per cent of unsaturation but also on the type of unsaturation and chain length. Moreover, the threshold level of unsaturated fatty acids for *E. coli* and *S. typhimurium* may be slightly different. Such lysis has been reported previously for some unsaturated fatty acid auxotrophs of *E. coli* (Esfahani *et al.*, 1969, 1971) and the effect of temperature on cellular lysis indicated that lysis is due to decrease in the fluidity of the membrane. It is quite remarkable that under both these conditions *i.e.*, growth in the presence of elaidic acid and after temperature shift, the unsaturated:saturated fatty acids ratio is the same and very low, 0·32. It is expected that under such conditions membrane fluidity will be reduced considerably. The results clearly suggest that transport of uridine is very much influenced by the ratio of unsaturated and saturated fatty acids. *E. coli* cells starved for fatty acids become leaky before cellular lysis (Overath *et al.*, 1971; Nunn and Cronan, 1974). So it may be argued that lack of accumulation is due to leakage of accumulated metabolite out of the cell, although to minimise such a possibility pulse labelling experiment was carried out. The main objective of this type of experiments was to study the differential effect of supplementation with *cis* and *trans*-unsaturated fatty acids. Our results indicate that the net rate of transport increases on supplementation with *cis*-unsaturated fatty acids as compared with that of *trans*-unsaturated ones except in the case of linolelaidic acid (figure 2) which resembles *cis* fatty acids so far as the physical properties are concerned. In case, the drastic reduction in the rate of transport is due to the leakage in the membrane, it has to be assumed that supplementation with *cis*-fatty acids or linolelaidic acid repairs the leakage of the membrane. So the apparent leakage can be attributed to the ratio of unsaturated and saturated fatty acids. However, the experiments described in figure 3 have been carried out with cells growing exponentially in fatty acid supplemented media and the question of lysis of those cells does not arise and thus the possibility of leakage is minimum. Those cells also exhibit remarkable difference in the rate of transport of uridine depending on the nature of the fatty acid present in the growth media. These facts suggest that uridine transport across the cell membrane depends on physical properties of the membrane lipids which is influenced at least to some extent by the ratio of saturated and unsaturated fatty acids. Supplementation with linolelaidic acid allows the cells to recover uridine transport. The unsaturated and saturated fatty acids ratio of cellular lipid of cells grown in linolelaidic acid is 1·28, higher than elaidic acid grown cells. The ratio of unsaturated fatty acid and saturated fatty acid, however, provides a gross idea about the membrane fluidity as the chain length of the fatty acids also contributes to the state of membrane fluidity. For example, although the unsaturated and saturated fatty acids of cells grown in the presence of linoleic acid is 0·71, it can provide suitable environment (proper fluidity) for the functioning of the uridine transport system. On the other hand, palmitelaidic acid grown cells having unsaturated and saturated fatty acids ratio of 0·97 do not manifest the normal rate

of uridine transport or respiration. Esfahani *et al.* (1972) have studied the effects of phospholipids on succinic–ubiquinone reductase activity of *E. coli* membranes. They have demonstrated that the transition of Arrhenius plot of succinic ubiquinone reductase activity is dependent on the nature of the polar group of ·phospholipids. Overath *et al.* (1970) studied the transition temperature of a number of membrane associated functions and correlated the biological function with the physical state of the cell membrane. Our results not only indicate the importance of the physical state of the membrane but also suggest that the lipid composition surrounding the membrane proteins, besides the fluidity, is also involved in regulating membrane function. At 37°C cells grown in the presence of *cis*-unsaturated fatty acids do not exhibit the same extent of activity of different biological functions tested. Cardiolipin has been implicated to play an important role in mitochondrial respiration (Rattery *et al.*, 1975). The cytoplasmic petit mutants of yeast (Jakovcic *et al.*, 1971) and respiratory deficient (rd3) mutant of *Aspergillus niger* (Mandal *et al.*, 1978) which have poor respiratory capacity contain significantly decreased amount of cardiolipin. Under the present set of experiments cardiolipin content is significantly low in cells grown at non-permissive temperature and in the presence of elaidic acid. However, cardiolipin content of palmitelaidic acid grown cells is comparatively high, 17·6% as opposed to 4·7% and 6·2% in the earlier two cases. Hence it seems that the cardiolipin content alone cannot regulate the activity of respiratory enzymes and fluidity parameter of cells may also be involved with the respiratory activity of the membrane. Lipid profiles of the mutant of *S. typhimurium* grown under different conditions support the idea that membrane function is greatly influenced by membrane structure specially the lipid environment. Study of transport of a number of metabolites and the use of a variety of fatty acids may be useful for this purpose.

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Use of epoxysepharose for protein immobilisation

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Abstract. Epoxy Sepharose, an activated affinity matrix which has been used for immobilisation of carbohydrates has been tried for immobilisation of proteins. Under normal conditions of coupling at neutral or alkaline pH proteins do not couple to epoxy Sepharose. However, a very high salt concentration during coupling allows the binding of proteins to epoxy Sepharose at a pH as low as 8·5. Increasing ionic strength and/or pH facilitates the binding. The bioactivity of the proteins is not destroyed by the immobilisation. This matrix, unlike cyanogen bromide-Sepharose, retains its ability to bind albumin by 80–90% even after 60 days of storage in aqueous suspension at 4°C. Its capacity to bind proteins is comparable to that of cyanogen bromide–Sepharose.

Keywords. Epoxy Sepharose; affinity matrix; protein immobilisation.

Introduction

Use of affinity techniques has become a routine laboratory method in biochemical research. Widely used activated solid supports have been cyanogen bromide (CNBr)-Sepharose for protein coupling and epoxyactivated Sepharose for carbohydrates.

Preparation of CNBr-Sepharose in a laboratory involves use of pungent and poisonous cyanogen bromide (fresh) and an activation procedure which is hazardous. In addition, the activated material so obtained has a very short life and needs to be used for coupling within couple of minutes (Axen *et al.*, 1967; Jakoby and Wilchek, 1974). The other activated Sepharose, namely, epoxy Sepharose (EPS) is used for coupling of ligands through hydroxyl groups (Sundberg and Porath, 1974; Vretblad, 1976). This support has been characterised to be capable of binding to amino, hydroxyl and sulphydral groups (Vretblad, 1976; Landt *et al.*, 1978; Simons and Vander Jagdt, 1977), and yet this has never been used earlier for protein coupling. A chance observation that EPS bound well to cytochrome C in concentrated sodium carbonate solution prompted us to investigate the coupling of proteins to EPS. In the present paper we report on the use of EPS for the immobilisation of proteins.

Materials and methods

Trypsin, albumin, haemoglobin, trypsin inhibitor and butanedioldiglycidyl ether were all purchased from Sigma Chemical Co., St. Louis, Missouri, USA. All other reagents used were analytical reagent grade. Sepharose 4B was a product of Pharmacia, Sweden.

Preparation of EPS

EPS was prepared by a standard procedure (Axen *et al.*, 1967). Briefly 40 g of Sepharose 4B was washed well with distilled water and suspended in 2 M sodium hydroxide (40 ml). Butanedioldiglycidyl ether (10 ml) was added and the suspension was stirred overnight at room temperature. The suspension was then filtered on a sintered funnel, washed extensively with water until the filtrate was neutral (2 litres of water), then with acetone (200 ml) and finally with 500 ml of water. The gel obtained was stored as aqueous suspension at 4°C for further use.

Binding of proteins and tryptophan to EPS

EPS (200–300 μl) in a 3 ml test tube was washed thoroughly with the required buffer and centrifuged to obtain a compact pellet. This pellet was dispersed in 100 μl of buffer and protein solution (200 mg/ml albumin, 100 mg/ml haemoglobin, 75 μmol tryptophan) taken in the same buffer (100 μl). This was intermittently vortexed and reaction was allowed to take place at room temperature. The suspension was diluted with 2 ml of phosphate buffer (0·2 M, pH 6·5) and spun. The optical density of the clear supernatant was measured. As control, Sepharose (200–300 μl) with protein and buffer was also incubated and processed identically. The decrease in the absorbance between control and experimental sample was used to quantitate the protein bound to the matrix.

Preparation of immobilised oLH antisera on Sepharose

1 ml of ovine leutinising hormone (oLH) antisera raised in the monkey was diluted to 5 ml with water and the immunoglobulin fraction from this antisera was precipitated by adding 5 ml of 25% polyethylene glycol at 4°C. The precipitate was spun at 5000 g for 30 min and the supernatant discarded. The pellet was collected and dissolved in 3 ml of 1 N dipotassium hydrogen phosphate (pH 9·0). This immunoglobulin G (IgG) enriched fraction was coupled to 2 ml of freshly prepared EPS equilibrated with the above buffer. The reaction was carried out at room temperature under mild shaking. The gel was washed well with water and stored at 4°C for further use. The extent of binding as measured by the decrease in the absorbance of the supernatant was about 70%.

Binding of trypsin to matrix bound trypsin inhibitor

Trypsin inhibitor coupled to EPS (4 mg trypsin inhibitor on 200 μl of EPS) was reacted with 300 μl of trypsin (1·65 mg) in 0·1 M sodium bicarbonate solution for 30 min. The reaction mixture was diluted with 2 ml water and the absorbance of the supernatant measured at 280 nm. The decrease in the A_{280} of the supernatant

was used to calculate the amount of trypsin bound to the matrix. As control trypsin (1·65 mg) was incubated with Sepharose (200 μl) and processed identically.

Removal of oLH antibodies from ovine follicle stimulating hormone antisera

oLH coupled to EPS was washed thoroughly with water (4 mg oLH coupled to 2 ml EPS) and mixed with 5 ml of ovine follicle stimulating hormone (oFSH) antisera. The mixture was initially shaken overnight at room temperature. The supernatant from this mixture was collected. The binding of [I]–oLH to this antiserum before and after treatment was done to assess the extent of removal of the oLH antibodies.

Determination of enzyme activity

Enzyme activity of glucose oxidase coupled to EPS was determined by the standard procedure. The formation of colour on incubation with glucose, peroxidase and *o*-dianisidine was taken as a positive indication for the presence of activity. Quantitative analysis of the enzyme activity was not undertaken.

Results

Binding of haemoglobin to EPS at alkaline pH conditions are shown in table 1. The coupling of the protein is insignificant even at pH 12 and nothing at pH values below 11, clearly demonstrating that EPS does not bind to proteins under normal conditions of coupling. Similar results were also obtained when coupling of albumin was attempted.

Table 1. Binding of haemoglobin to EPS at pH 10–12.

pH*	Haemoglobin bound mg/ml gel
12	1·5
11	0·0
10	0·0

* Reaction was carried out in 0·01 M, 0·001 M and 0·0001 M sodium hydroxide, respectively. 20 mg of haemoglobin was reacted with 0·5 ml of EPS. Time of reaction was 18 h.

Figures 1 and 2 show the effect of ionic strength on the binding of proteins (albumin and haemoglobin). Increasing salt concentration facilitates the coupling (figure 1). Increasing the ionic strength by neutral salts also increases the binding markedly (figure 2), demonstrating that the coupling is facilitated by increasing ionic strength (salt concentration).
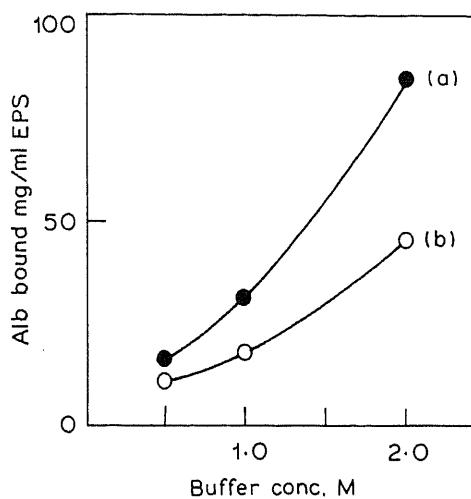
Figure 1. Effect of buffer concentration on binding of albumin to EPS. Curve (a), 2 M K₂HPO₄ pH 9; Curve (b), 2 M Na₂CO₃ pH 10·6. Time of reaction in both cases was 2 h at room temperature.
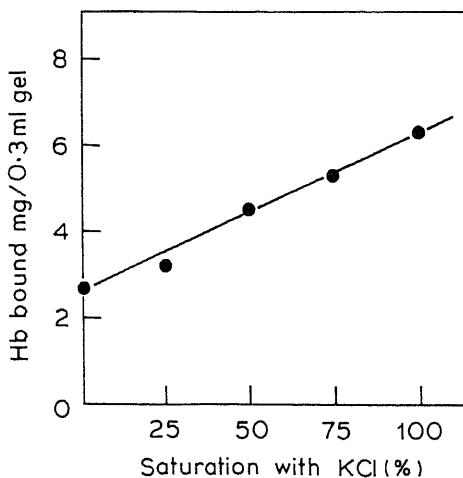
Figure 2. Effect of salt concentration on binding of haemoglobin to EPS: Conditions of reaction: 2 M sodium carbonate, buffer pH 9·0 and time of reaction 1 h.

Figures 3 and 4 show the effect of pH and time on the coupling efficiency of proteins to EPS. It is evident from the figures that higher pH and longer time increases the efficiency of binding markedly.

Binding of haemoglobin as a function of its concentration is shown in figure 5 (curve a). It indicate that the binding of the protein is dependent on its concentration during coupling (curve a). Within the range of concentrations used, the haemoglobin bound as a per cent of each concentration remained essentially constant (curve b).
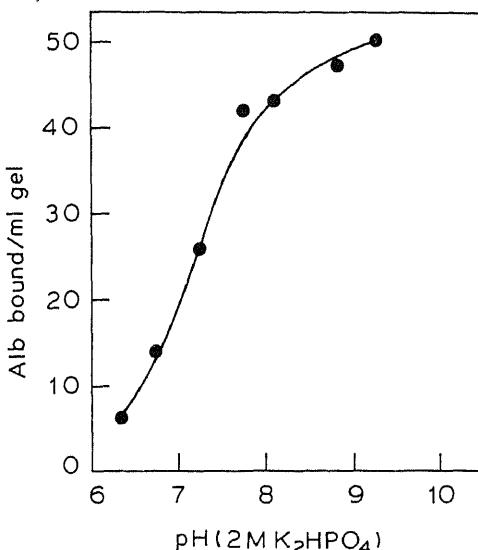
Figure 3. Effect of pH on the binding of albumin to EPS: Conditions of reaction: 2 M potassium phosphate buffer (pH ~9) and time of reaction 2 h.
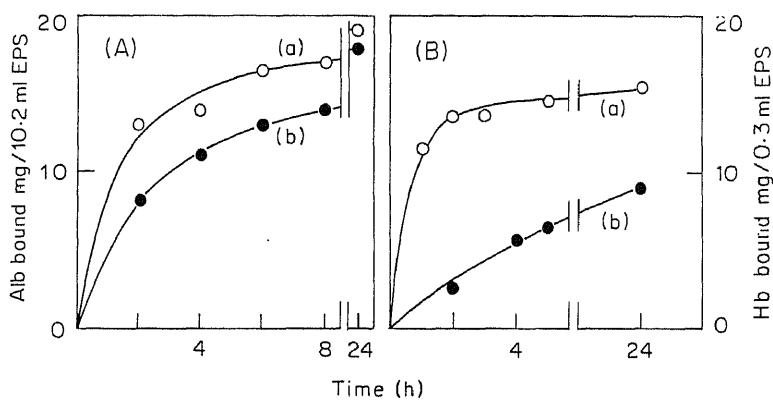
Figure 4. Kinetics of binding of proteins to EPS. **A.** Binding of albumin. Curve (a), 2 M potassium phosphate, pH 9; curve (b), 2 M potassium phosphate, pH 8. **B.** Binding of haemoglobin. Curve (a), 2 M sodium carbonate pH 9 saturated with KCl; curve (b), sodium carbonate pH 8, saturated with KCl.

Coupling efficiency of tryptophan to EPS is depicted in figure 6. Saturation of binding occurs at 40 μM concentration of tryptophan and the capacity of the gel as measured by this method is 8–10 μmol/ml. Unlike in the case of proteins the binding of tryptophan to EPS was found to be ionic strength independent.

Table 2 presents the binding of normal sheep serum (NSS) and the IgG fraction. of NSS and other proteins to EPS. The binding of IgG is almost complete while the binding of the NSS is nearly 50%. It is also seen that binding of proteins can occur
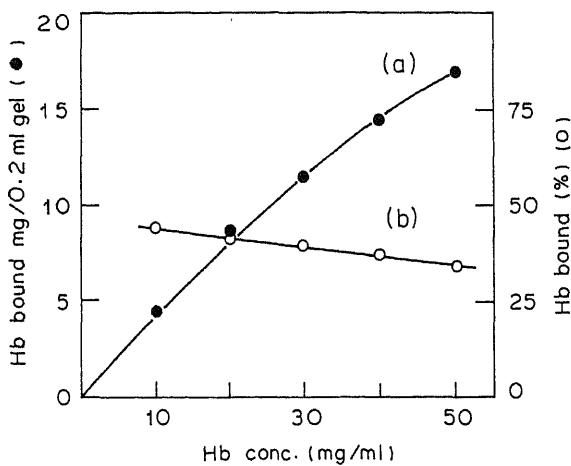
Figure 5. Binding of haemoglobin as a function of its concentration: Conditions of reaction: 1 M potassium phosphate buffer, pH 9, time of reaction 20 h at room temperature. Curve (a), Haemoglobin bound/0·2 ml gel; curve (b), per cent of haemoglobin bound.
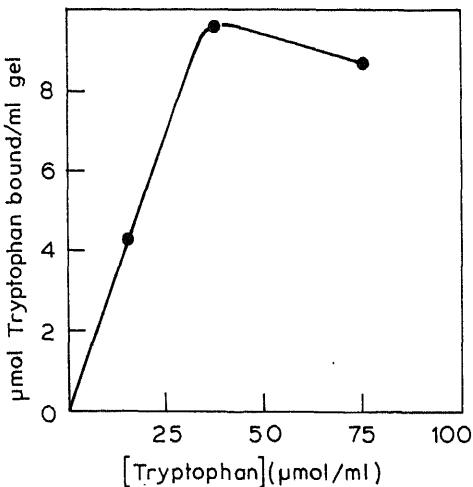
Figure 6. Binding of tryptophan to EPS: Buffer–0·1 M carbonate pH 10·5; time, 20 h at room temperature. For all calculations a molar extinction coefficient of 5400 at 280 nm was employed.

at pH around 9. In these proteins, enzyme activity of glucose oxidase, trypsin binding activity of trypsin inhibitor, immunoactivity of oLH were tested after coupling to EPS and found to be intact, clearly indicating retention of activity after coupling. Quantitative analysis of the binding data indicated that trypsin inhibitor–EPS could bind 1 mg of trypsin. Likewise the oLH antibody coupled to EPS bound 800 μg of oLH (the capacity of the antisera coupled as measured by

Table 2. Coupling of proteins to epoxysepharose.

	Protein used										
	Glucose oxidase		Trypsin inhibitor[a]		Ovine LH[b]		IgG	Trypsin	oLH a/s[c]	Sheep IgG	Sheep serum
pH	8·8	9·6	9·5	10·6	9·5	10·6	10·0	9·0	9·0	10·6	10·6
Protein added (mg)	12·0	12·0	9·5	9·5	7·0	7·0	5·0	20·0	25·0	7·0	16·0
Protein bound (mg)	2·6	0·7	4·2	4·6	1·0	5·7	4·5	4·0	18·0	6·0	8·0

[a] The immobilised trypsin inhibitor bound 1 mg trypsin.
[b] This oLH was used for the removal of the contaminating oLH antibodies from oFSH a/s. The removal of the oLH antibodies was quantitative.
[c] The insolubilised antiserum removed quantitatively the oLH contamination (800 μg) from oFSH preparation (10 mg).
In all these cases 0·3 ml of EPS was used for coupling in 1 M potassium phosphate, overnight.

Scatchard analysis was 500–600 μg). Thus the binding activity of trypsin inhibitor is partially lost (50%) on immobilisation whereas the immunological activity of the antibody is retained completely after immobilisation on EPS.

Figure 7 shows the stability of the EPS and CNBr-Sepharose stored in suspension. EPS retains to a large extent the capacity to bind to albumin even after 90 days, whereas CNBr-Sepharose loses it within a couple of days.
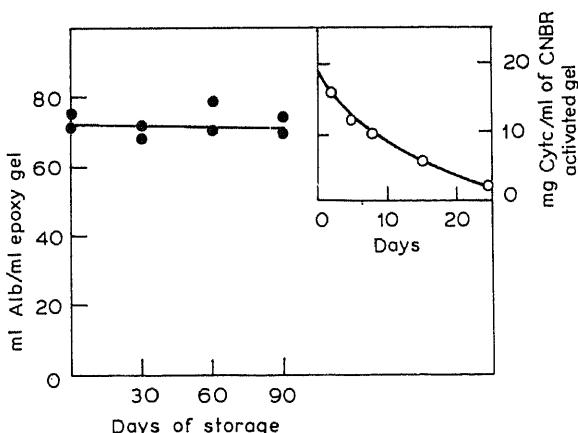
Figure 7. Effect of storage of EPS and CNBr-Sepharose (in aqueous solution at 4°C) on protein binding capacity. Binding was carried out as described under methods. Albumin and cytochrome C were used for EPS and CNBr-Sepharose, respectively. Inset shows the stability of CNBr-Sepharose.

Discussion

The data presented demonstrates that EPS can be used for coupling of proteins to Sepharose matrix. So far its use has been limited to coupling carbohydrates through their hydroxyl groups. This could presumably be due to the fact that under normal conditions of coupling (pH 8–9, 0·1 M sodium bicarbonate) EPS does not bind to proteins (table 1). The parameters which appear to facilitate remarkably the coupling are ionic strength and pH (figures 2 and 3). High ionic strength facilitates the coupling further (figure 4). The coupling of proteins to the matrix can be effected at pH values as low as 8–9, where most proteins retain their structure or biological activity. The capacity of the matrix for binding albumin and haemoglobin is quite comparable to that of CNBr–Sepharose.

Albumin and haemoglobin coupled to EPS could not be dissociated by 5 M potassium iodide or glycine HCl buffer pH 2·8. Thus coupling of proteins to the matrix could be either through the hydroxyl group of serine or threonine, or the α or ϵ amino groups. As yet there is no clear proof to implicate any one of these groups in this reaction. However, it is observed (Murthy, G. S., unpublished results) that the amino groups of albumin and other proteins can be completely blocked by adding the epoxy reagent, favouring the amino group as the possible point of attachment.

Coupling of proteins to EPS does not cause destruction of activity of the proteins (table 2). This clearly indicates the essential feasibility of using this matrix for affinity purification of biologically active molecules.

The stability of EPS in suspension is very much more than that of CNBr-Sepharose (figure 7). Its preparation is also very easy. For these reasons preparation and use of EPS in affinity techniques is more convenient than that of CNBr-Sepharose in laboratories. Under conditions where the proteins are insoluble at relatively high ionic strength, EPS may be only of limited use.

The stability of the linkage between the epoxy group and the amino group is expected to be much stronger than that between the amino and the cyanate ester group (active groups in the CNBr-Sepharose). Thus it is possible that the linkage between the protein and EPS could be stronger than that between CNBr-Sepharose and protein.

EPS can be easily used for the preparation of AH-Sepharose, CH-Sepharose etc by coupling with appropriate ligands, by procedures described for coupling of trytophan. Preparation of lysine-Sepharose, arginine-Sepharose and histidine-Sepharose have been done using the above procedure (Murthy, G. S., unpublished results). Thus EPS provides a versatile affinity matrix which can be easily prepared in the laboratory and used for both protein and carbohydrate coupling.

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Interaction of proteins with detergents: Binding of cationic detergents with lysozyme*

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Abstract. Binding studies of cationic detergents such as cetyl trimethylammonium bromide, Cetylpyridinium bromide and dodecyl trimethylammonium bromide with lysozyme were carried out by equilibrium dialysis, ultraviolet difference and circular dichroism techniques at 25°C. Binding isotherms at pH 5·0, 7·0 and 9·0 show cooperative binding at all concentrations of detergents and the number of available binding sites in lysozyme increases with pH. Gibbs' free energy of binding calculated on the basis of Wymans' binding potential concept increases with pH indicating increased binding strength at higher pH. The ultraviolet difference spectra of the detergent complexes with lysozyme at pH 7·0 and 9·0 in the region of 250–300 nm indicate the involvement of aromatic amino acid residues as probable binding sites and also the carboxylate groups since the binding is cooperative. The circular dichroism spectra also indicate the involvement of aromatic amino acid residues in the binding of these detergents. This is substantiated by the decrease in the intensity of the aromatic positive bands in the near ultraviolet region. The increase in the magnitude of $[\theta]_{222\,nm}$ values in the far ultraviolet region with the increase in the concentration of the detergent in the complex indicates conformational changes resulting in an increase of α-helical content producing a more ordered structure of lysozyme. These binding studies show that at pH 7·0 and 9·0, hydrophobic interactions play a major role, while at pH 5·0 only electrostatic interactions play prominent role in the binding of these detergents.

Keywords. Lysozyme; cationic detergents; cooperative binding; helical content.

Introduction

The interactions of proteins with ions and neutral molecules or a variety of ligands in general have been widely investigated and have been reviewed by Steinhardt and Reynolds (1969). Of them, the interaction studies of ionic detergents with proteins have received much attention (Jones, 1975) and it is generally accepted that the binding of such detergents to proteins occurs by a combination of electrostatic and hydrophobic interactions. The relative importance of these types of interactions can be assessed by a study of the binding of a typical globular protein like lysozyme

with a series of detergents having hydrocarbon moieties of varying lengths and different polar head groups. Such a study will serve as a model since lysozyme has been well characterised physicochemically (Johnson and Philips, 1965; Rupley 1967; Rupley and Gates 1967) and its 3-dimensional structure well established (Blake *et al.*, 1965, 1967; Philips 1966).

The binding studies of lysozyme with various detergents have been carried out by several workers using different techniques (Jones 1983; Jones and Manley, 1979, 1980, 1981, 1984; Jones *et al.*, 1981; Imoto *et al.*, 1979; Hayashi *et al.*, 1968a, b; Fukushima *et al.*, 1982, Kato Akco *et al.*, 1984) under a variety of conditions, in order to determine the thermodynamics of binding, the nature and number of available binding sites, the types of interactions involved and the conformational changes accompanying such interactions.

In the present investigation, we describe the interaction of lysozyme with cationic detergents such as cetyl trimethylammonium bromide (CTAB), dodecyl trimethyl-ammonium bromide (DTAB) and cetyl pyridinium bromide (CPB) at various concentrations and pH using equilibrium dialysis technique. The thermodynamic parameters of their binding are evaluated. The nature of binding sites and the types of interactions involved are determined by ultraviolet (UV) difference spectra. The conformational changes in the enzyme accompanying the binding of these detergents are followed by the circular dichroism (CD) technique. However, the enzymatic activity of lysozyme due to the binding of these detergents has not been undertaken in this study.

Materials and methods

Hen egg-white lysozyme (3X crystallized, lot No. 6876). CTAB, DTAB, CPB, L-tryptophan (Trp), L-tyrosine (Tyr)-HCl and L-phenylalanine (Phe) from Sigma Chemical Company, St. Louis, Missouri, USA were used. All other reagents used were of analytical grade and the solutions were prepared using double distilled water. For equilibrium dialysis, membrane tubing from Union Carbide Corporation, Chicago, Illinois, USA was used. UV-visible spectrophotometer (Carl Zeiss) was used to measure the concentration of lysozyme and detergents. $A_{1\,cm,\,280\,nm}^{1\%} = 26 \cdot 4$ for lysozyme (Sophianopoulos *et al.*, 1962) was used to calculate the concentration of lysozyme.

The concentrations of detergents are determined by extracting their bromophenol blue complex in chloroform as described earlier (Mukerjee and Mukerjee, 1962, Ray and Chatterjee, 1967).

Jasco J-20 spectropolarimeter, calibrated with (+)-10 Camphor sulphonic acid (De Tar, 1969) was used to record the CD spectra. Beckmann spectrophotometer Model-25 was used to record the UV difference spectra.

In the equilibrium dialysis experiments, 5 ml of 0·2% lysozyme ($1 \cdot 4 \times 10^{-4}$ M; molecular weight 14,400) was dialysed against 10 ml of detergent solution at various concentrations in the same buffer as described earlier (Subramanian and Venkatappa, 1982). The time required to attain equilibrium was found to be 24 h at 25°C. The binding ratio 'r', the number of mol of the detergent bound per mol of the protein, at various free detergent concentrations $[D]$ was calculated in a manner similar to Rosenberg and Klotz (1961).

The binding studies were carried out in 3 buffer systems: 0·01 M sodium acetate buffer, pH 5·0, 0·01 M phosphate buffer, pH 7·0 and 0·01 M glycine buffer, pH 9·0 and at varying detergent concentrations with a fixed protein concentration of 1.4×10^{-4} M. The critical micelle concentrations (CMCs) of the detergents are 9.2×10^{-4} M for CTAB, 1.56×10^{-2} M for DTAB and 5.8×10^{-4} M for CPB (Mukerjee and Mysels, 1971). The free detergent concentrations used were well below and above their CMCs. The concentrations of the buffers used in these studies are such that no unequal distribution of free detergent molecules due to the Donnan effect occur during the equilibrium dialysis. Binding studies were carried out at pH 5·0, 7·0 and 9·0 in the case of CTAB and CPB, while with DTAB the studies were carried out only at pH 9·0. In order to investigate the effect of ionic strength on the binding of detergents to lysozyme, experiments were carried out only at pH 9·0 with 0·05 M glycine buffer.

The CD spectra of lysozyme and its complex with the detergents were measured at a protein concentration of 2.3×10^{-5} M in a 0·5 mm cell from 250–200 nm and 3.5×10^{-5} M in a 10 mm cell from 320–250 nm at 25°C. The results are represented as molar residue ellipticity, $[\theta]_\lambda$ in deg cm^2 dmol^{-1}, calculated in a manner similar to that of Adler *et al.* (1973) taking a value of 111 as mean residue weight for lysozyme (Warren and Gordon, 1970).

The UV difference spectra of lysozyme-detergent complex were recorded at a fixed protein concentration of 7.0×10^{-5} M in all cases and varying the detergent concentration. Both UV difference and CD spectra of the complex were recorded 3 h after mixing.

Results and discussion

Binding isotherm

The binding isotherms obtained for the interaction of lysozyme with CTAB and CPB at pH 5·0, 7·0 and 9·0 and with DTAB at pH 9·0 are shown in figure 1 as a plot of *r vs* log $[D]$ and they follow cooperative binding at all pH. The binding isotherms have been interpreted in terms of the Wyman's binding potential concept (Wyman 1965) as adopted by Jones and Manley (1979, 1980, 1981) and subsequently by others in the binding studies (Subramanian *et al.*, 1983, 1984). From the plots of *r vs* log $[D]$, the apparent free energy of binding, ΔG_r is calculated in a manner similar to that of Jones and Manley (1981) from which the apparent Gibbs free energy of binding per bound detergent ion is computed from the relation, $\Delta G_r = \Delta G_a / r$. The results of these interaction studies with CTAB and CPB at various pH and with DTAB at pH 9·0 only are shown in the plot of ΔG_r *vs r* (figure 2), from which the maximum values of ΔG_r are calculated.

It has been observed that the binding ratios in each case increase with pH. The increase in the number of available binding sites with pH is in accordance with the fact that there is a decrease in the net positive charge on lysozyme with the increase of pH. The number of binding sites, Gibbs' free energy of binding per bound detergent ion, ΔG_r, and the binding constant K, at various pH and ionic strengths are summarized in table 1.

As the free detergent concentration approaches CMC, the binding isotherms show a marked increase in the average number of detergent ions bound per mol of
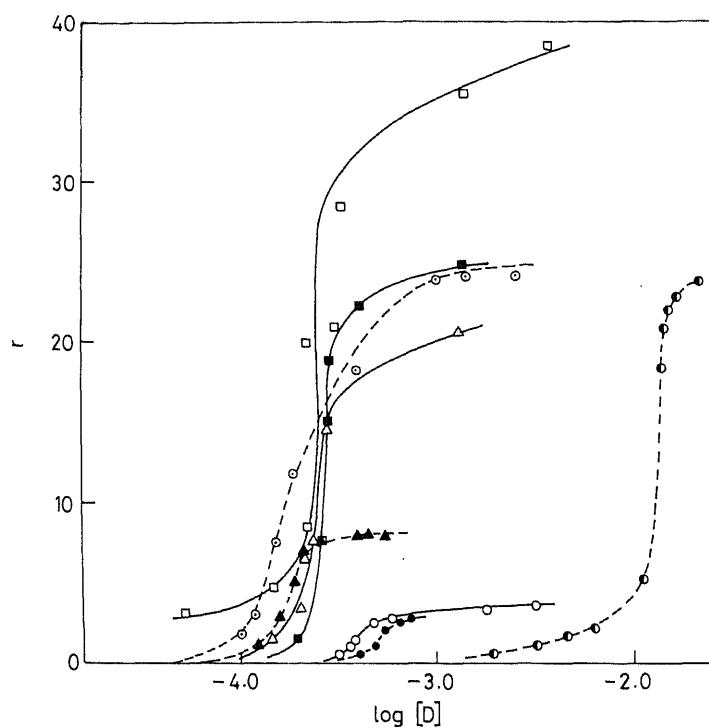
Figure 1. Binding isotherms for the interaction of lysozyme with detergents at 25 °C: *r vs* log [*D*].

   (i) Binding of CTAB: (□), 0·01 M Glycine buffer, pH 9·0; (■), 0·05 M Glycine buffer, pH 9·0; (△), 0·01 M Phosphate buffer, pH 7·0; (○), 0·01 M Acetate buffer, pH 5·0.

   (ii) Binding of CPB: (⊙), 0·01 M Glycine buffer, pH 9·0; (▲), 0·01 M Phosphate buffer, pH 7·0; (●), 0·01 M Acetate buffer, pH 5·0.

   (iii) Binding of DTAB: (◐), 0·01 M Glycine buffer, pH 9·0.

Table 1. Thermodynamic parameters for the binding of detergents with lysozyme at 25 °C.

Detergent	Buffer	pH	Total number of binding sites 'n'	ΔG_r max[a] (k cals mol^{-1})	K[b]
	Acetate buffer (0·01 M)	5·0	4	−4·62	2400
	Phosphate buffer (0·01 M)	7·0	20	−5·15	5900
CTAB	Glycine buffer:				
	(i) 0·01 M	9·0	38	−5·95	23000
	(ii) 0·05 M	9·0	24	−5·29	7500
DTAB	Glycine buffer (0·01 M)	9·0	24	−3·43	300
	Acetate buffer (0·01 M)	5·0	3	−4·52	2060
	Phosphate buffer (0·01 M)	7·0	8	−5·17	6170
CPB	Glycine buffer (0·01 M)	9·0	24	−5·56	11920

[a] $\Delta G_{r,\ max}$ is the maximum value of ΔG, computed from the plot of *r vs* log *D*.
[b] Binding constant *K* values are calculated from $\Delta G_{r,\ max}$.
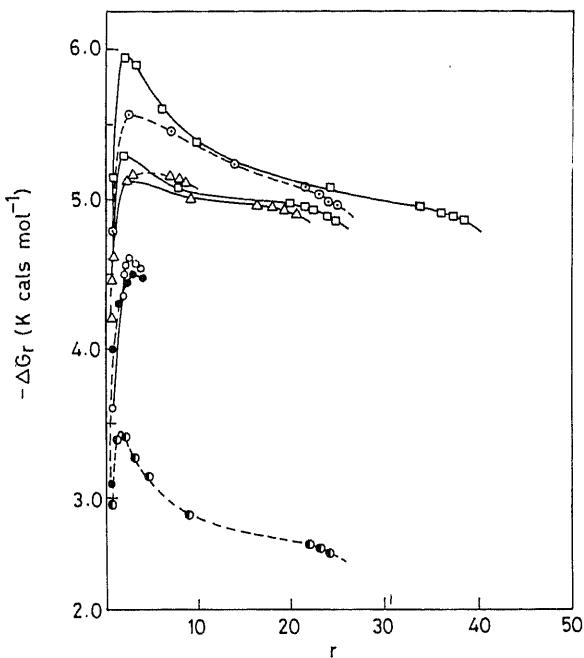
Figure 2. Plot of ΔG_r (k cals mol^{-1}) *vs* r for the interaction of lysozyme with the detergents at 25°C. Same notations as in figure 1.

the protein over a relatively small range of detergent concentration and reaches a limiting value. Thus cooperative binding reaches a limit when lysozyme becomes saturated with the detergent. This is in accordance with the observation by earlier workers on the interaction of lysozyme with sodium dodecyl sulphate (SDS) (Jones *et al.*, 1981).

UV difference spectra

The UV difference spectra of lysozyme-CTAB complex at pH 5·0 (0·01 M) showed no characteristic peaks in the aromatic region while a distinct peak was observed for the complex at pH 7·0 (0·01 M) around 295–297 nm. The intensity of the peak increased with the increase in the concentration of CTAB in the complex (figure 3). The UV difference spectra of the complex at pH 9·0 (0·01 M) showed two peaks one around 295–297 nm and the other at 250 nm (figure 4). Here again, the intensity of both the peaks increased with the increase in the concentration of CTAB in the complex upto a certain limit and then showed a decrease.

The UV difference spectra of L-Trp-CTAB complex at pH 9·0 (0·01 M) showed a strong peak at 292 nm and a weak one around 282–283 nm (figure 5). The UV difference spectra of L-Tyr-CTAB complex at pH 9·0 (0·01 M) showed a peak around 245–246 nm in addition to the one observed at 285–286 nm (figure 6). No significant UV difference spectra were observed for these complexes at pH 7·0 and 5·0.

The UV difference spectra of lysozyme–CPB complexes were recorded in the region of 270–350 nm only, as it was not possible to record them below 270 nm
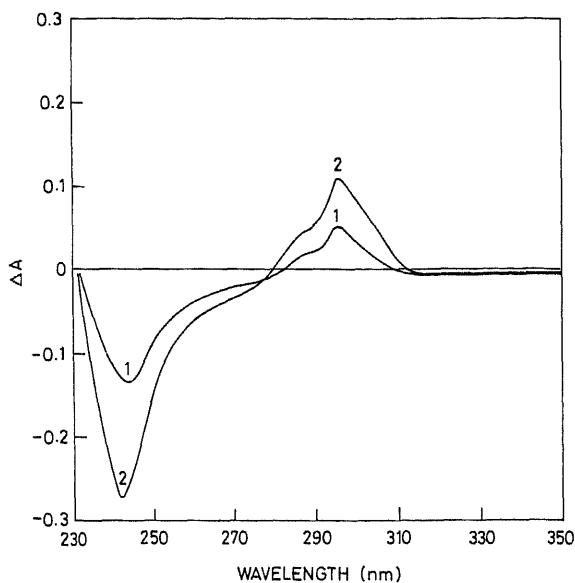
Figure 3. UV difference spectra of 7×10^{-5} M lysozyme in 0·01 M phosphate buffer, pH 7·0 produced by CTAB. (1), $1·37 \times 10^{-3}$ M and (2), $2·74 \times 10^{-3}$ M.
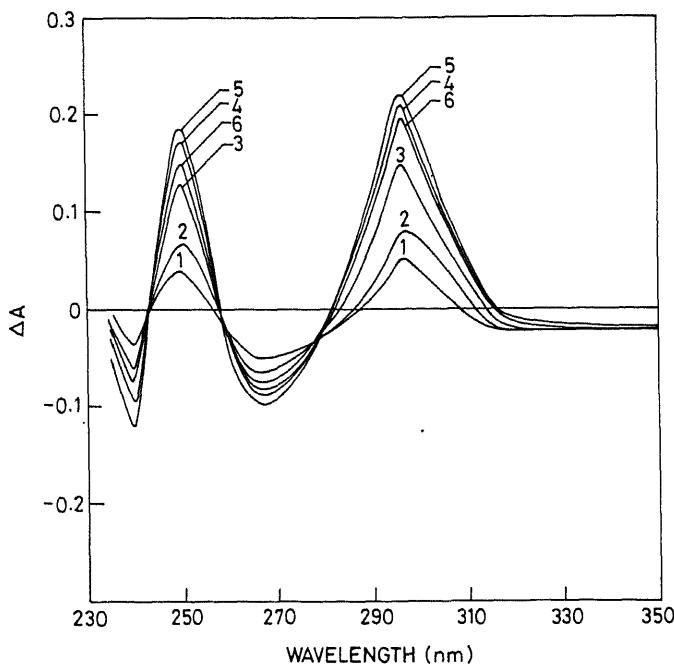
Figure 4. UV difference spectra of 7×10^{-5} M lysozyme in 0·01 M glycine buffer, pH 9·0, produced by CTAB. (1), $0·685 \times 10^{-3}$ M; (2), $0·96 \times 10^{-3}$ M; (3), $1·371 \times 10^{-3}$ M; (4), $2·055 \times 10^{-3}$ M; (5), $2·74 \times 10^{-3}$ M and (6), $4·11 \times 10^{-3}$ M.
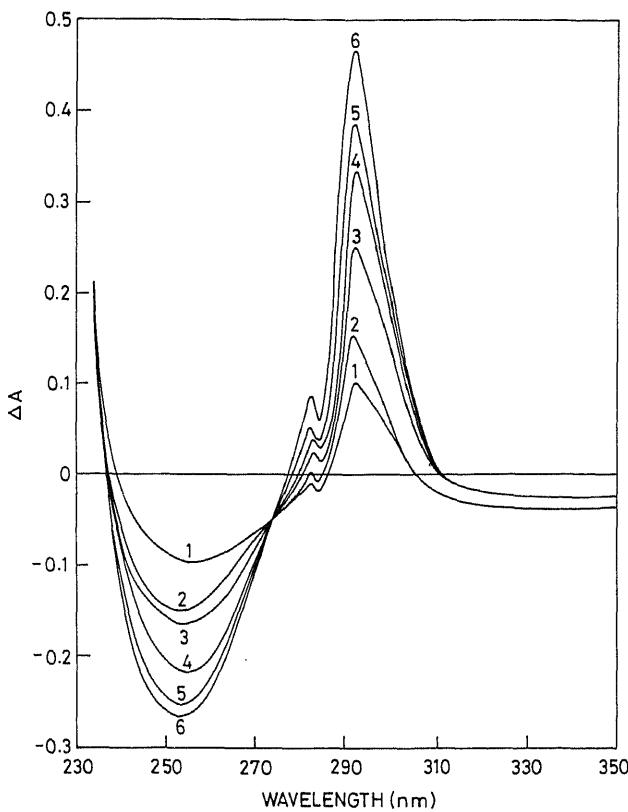
Figure 5. UV difference spectra of 5×10^{-4} M L-Trp in 0·01 M glycine buffer, pH 9·0 produced by CTAB. (1), $0·685 \times 10^{-3}$ M; (2), $0·96 \times 10^{-3}$ M; (3), $1·371 \times 10^{-3}$ M; (4), $2·055 \times 10^{-3}$ M; (5), $2·74 \times 10^{-3}$ M and (6), $4·11 \times 10^{-3}$ M.

since the detergent itself showed a very high UV absorbance in this region. At pH 5·0, no characteristic peak was observed in the aromatic region, while at pH 7·0 and 9·0, a distinct peak was observed around 295–297 nm, as in the case of lysozyme-CTAB complexes but with lesser intensity. The UV difference spectra of L-Trp-CPB complex at pH 9·0 showed a strong peak around 292–293 nm, while no peaks were observed in the case of L-Tyr-CPB complex at this pH.

The absence of peaks in the UV difference spectra in the aromatic region for the complexes of lysozyme either with CTAB or CPB at pH 5·0 indicates the non-involvement of aromatic amino acid residues in the interaction at this pH. However, the binding observed at this pH may be only due to the interaction of cationic head groups of the detergents with carboxylate anions on the lysozyme molecule involving mainly electrostatic interactions. With the increase in pH, more detergent ions bind at additional sites available on account of the ionization of side chain carboxyl groups of the enzyme, probably involving the rupture of some hydrogen bonds. The appearance of 295–297 nm peak in the UV difference spectra of the complexes of these detergents with lysozyme at pH 7·0 and pH 9·0 indicates the involvement of indole rings of tryptophan residues in addition to the carboxyl
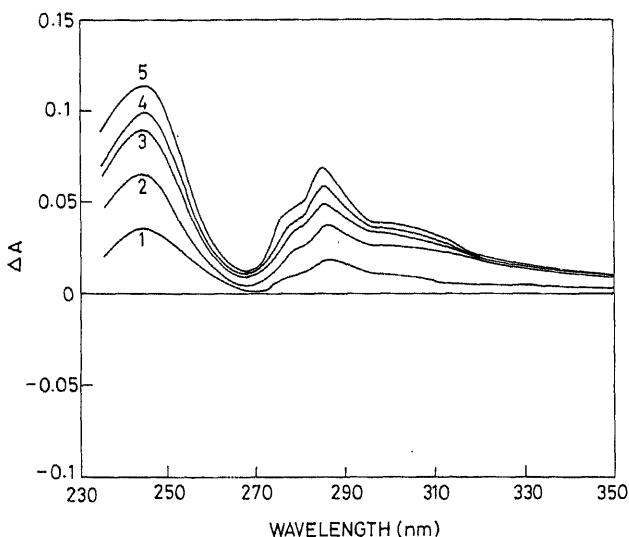
Figure 6. UV difference spectra of 5×10^{-4} M L-Tyr·HCl in 0·01 M glycine buffer, pH 9·0, produced by CTAB. (1), $0·685 \times 10^{-3}$ M; (2), $0·96 \times 10^{-3}$ M; (3), $1·37 \times 10^{-3}$ M; (4), $2·055 \times 10^{-3}$ M and (5), $2·76 \times 10^{-3}$ M.

sites. This is in accordance with the earlier observation of Hayashi *et al.* (1968) for the interaction of lysozyme with dimethyl benzyl myristyl ammonium bromide (DBMA), where they have shown that DBMA which acts as a competitive inhibitor for lysozyme binds at all the tryptophan residues lying on the surface and with one or two in the cleft by hydrophobic interactions.

The appearance of two peaks in the case of lysozyme-CTAB complex at pH 9·0 (0·01 M) as well as in the UV difference spectra of the complexes of CTAB with L-Trp and L-Tyr·HCl at the same pH indicates the involvement of both tryptophan and tyrosine residues along with the carboxyl groups in the binding of CTAB with lysozyme. The red shift of 4–5 nm in the 297 nm band of lysozyme-CTAB complex from that of L-Trp-CTAB complex at the same pH may be due to the binding of the CTAB at or near the tryptophan residues which are buried in the non-polar environment and also probably due to the partial ionization of a tyrosine residue in lysozyme at this pH. The UV difference spectrum for the ionization of tyrosine residues in lysozyme (Togo *et al.*, 1966) at alkaline pH showed two maxima, one at 295 nm and the other at 245 nm, their difference in molar extinction coefficients being 2650 and 13,200, respectively. So the appearance of an intense peak around 250 nm in the lysozyme-CTAB complex at pH 9·0 (0·01 M) may be due to the involvement of partially ionized tyrosine residues as the binding sites, while the appearance of only one peak in the UV difference spectra of lysozyme-CTAB complexes at pH 7·0 (0·01 M) is probably due to the binding of the detergent molecules only at tryptophan residues as tyrosines do not ionize at this pH.

The appearance of a strong peak around 292–293 nm in the UV difference spectra of L-Trp-CPB complex at pH 9·0 also indicates the involvement of tryptophan residues in the binding of CPB with lysozyme, and absence of any peak

in the UV difference spectra of L-Tyr-CPB complexes shows the non-involvement of tyrosine residues as binding sites at this pH.

The UV difference spectra of lysozyme-CTAB complex recorded at pH 9·0 at a higher ionic strength of 0·05 also showed two distinct peaks (figure 7) in the region 295–297 nm and 250 nm, however, with lesser intensities, the 250 nm peak decreasing to a larger extent than the one observed at a lower ionic strength of 0·01 at the same pH. This effect of ionic strength in decreasing the interaction of CTAB with lysozyme is supported by the fact that the binding isotherms also showed decreased binding at this ionic strength and pH. Accordingly there has been a decrease in the number of available binding sites and ΔG_r.
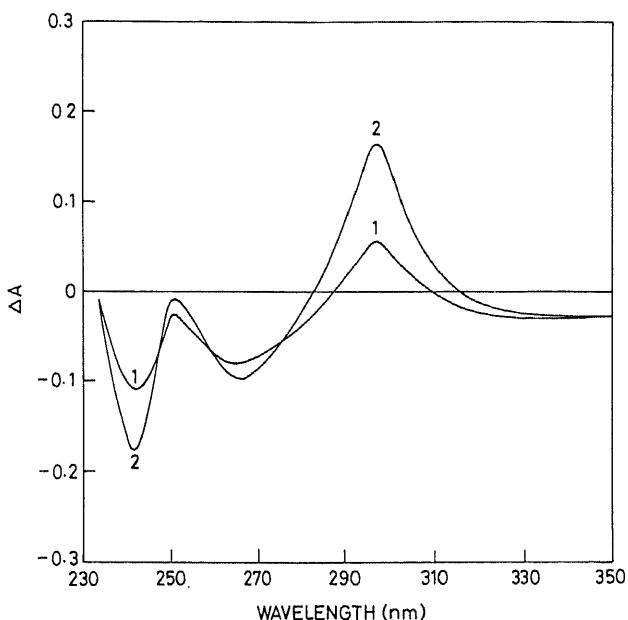
Figure 7. UV difference spectra of 7×10^{-5} M lysozyme in 0·05 M glycine buffer, pH 9·0 produced by CTAB. (1), $1·37 \times 10^{-3}$ M and (2), $2·74 \times 10^{-3}$ M.

A comparison of the results of the binding of CPB with those of CTAB to lysozyme indicates that only a lower number of binding sites are available to CPB at any pH. Accordingly, the magnitude of ΔG_r values shows a decrease in the case of CPB. The small difference in ΔG_r values at any given pH, for the binding of CTAB and CPB with lysozyme (table 1) indicates that electrostatic interactions play only a very minor role even though they possess different cationic head groups. The small difference may be attributed to the bulkiness of the polar head group in the case of CPB, since both the detergents have the same hydrocarbon chain length. The decreased binding in the case of CPB may also be due to the non-involvement of tyrosine residue in binding.

Although the extent of binding in the case of CPB is lower than that of CTAB, the observed high binding ratios at pH 9·0 in both the cases cannot be explained

with all the available carboxyl and aromatic amino acid residues in lysozyme. Moreover, the absence of peaks in the 240–300 nm region of the UV difference spetra of L-Phe-detergent complexes indicate the non-participation of any phenylalanine residues. So the observed high binding ratios can be explained as follows: the electrostatic interaction of the detergent with the carboxylate anions of lysozyme and the resultant binding are stabilized probably by the mutual affinity of the hydrocarbon chain of the adjacently bound detergent ion (Putnam, 1948) by non-polar forces which are presumed to be the same as those that bind the detergents to form micelles.

The UV difference spectra of DTAB with lysozyme and with model compounds L-Trp and L-Tyr·HCl at pH 9·0 follow a pattern similar to those of CTAB complex with lysozyme at pH 9·0 (0·01 M). The intensities of both the peaks at 250 and 297 nm of the lysozyme-DTAB complex were lower when compared with those of lysozyme-CTAB complex at the same pH. This indicates a lesser interaction between lysozyme and DTAB than with CTAB. Accordingly, the number of binding sites are lower for lysozyme-DTAB complex at pH 9·0 (0·01 M) than for lysozyme-CTAB complex at the same pH. A large difference in ΔG_r and K values have also been observed in the binding of these detergents with lysozyme. Thus these data suggest a stronger interaction for CTAB with lysozyme and a weaker interaction for DTAB. Since both CTAB and DTAB have the same cationic head groups, the contribution from the electrostatic interaction must be the same. Therefore, the very high binding energy and binding constant observed in the case of lysozyme-CTAB interaction are probably due to its longer hydrocarbon chain length than that of DTAB.

CD spectra

The CD spectra of native lysozyme show positive maxima at 293, 288, 282 and 278 nm and a broad negative band around 258–259 nm in the 320–250 nm region at all pH. The aromatic positive bands for the complexes of lysozyme with CTAB and CPB at pH 9·0 (0·01 M) decrease gradually and are replaced by an overall negative spectra with the increase in the concentration of the detergents in the complexes (figure 8a). The magnitude of molar ellipticity values around 257–259 nm decreases with the increase in the concentration of the detergents in the complex and also shows a gradual blue shift of 4–5 nm. The CD spectra of the complexes of lysozyme with these detergents recorded at pH 7·0 and 5·0 show similar patterns to those at pH 9·0, but with a lesser intensity of the aromatic bands.

In the far UV region of 250–200 nm the CD spectrum of lysozyme has an extremum at 208 nm and a shoulder at 222 nm. However, the magnitude of molar ellipticity $[\theta]_{222\,nm}$ values for the complexes of lysozyme with CTAB and CPB at pH 9·0 (0·01 M) increases upto certain concentrations of the detergents indicating an increase in the α-helical content (figure 8b). The α-helical content calculated by the method of Chen *et al.* (1972) increased from a fH value of 0·24 for the native lysozyme to a maximum value of 0·32 in the case of CTAB and 0·286 for CPB indicating more ordered structures. In this region, however, at pH 7·0 and 5·0, there was no detectable difference between CD spectra of native lysozyme and its complexes with these detergents.
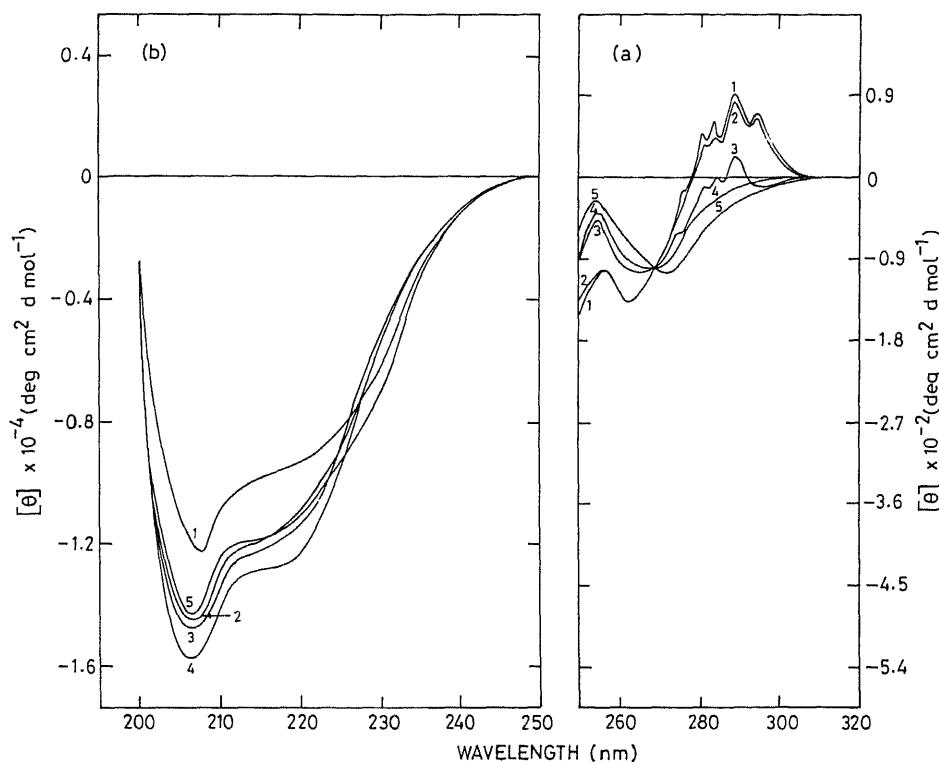
Figure 8. (a), Effect of CTAB on the CD spectrum of lysozyme in 0·01 M glycine buffer, pH 9·0 (320–250 nm region). (1), 0; (2), 0·274×10⁻³ M; (3), 0·683×10⁻³ M; (4), 0·891×10⁻³ M and (5), 1·095×10⁻³ M. Protein concentration: 3·5×10⁻⁵ M. (b), Effect of CTAB on the CD spectrum of lysozyme in 0·01 M glycine buffer, pH 9·0 (250–200 nm region). (1), 0; (2), 0·274×10⁻³ M; (3), 0·639×10⁻³ M; (4), 0·91×10⁻³ M and (5), 1·09×10⁻³ M. Protein concentration: 2·3×10⁻⁵ M.

The CD spectra of lysozyme-DTAB complex at pH 9·0 (0·01 M) in the 320–250 nm region showed a decrease in the aromatic bands in a manner similar to that of lysozyme-CTAB complex, but with a less pronounced effect. In the 250–200 nm region, the CD spectrum of lysozyme-DTAB complex with the detergent concentrations well below its CMC showed no change in the α-helical content. However, the CD spectra of the complex at and above its CMC cannot be recorded as the DTAB showed a high absorbance.

The CD spectra of lysozyme-detergent complexes in the 320–250 nm and 250–200 nm regions show that the detergents might bind with the indole rings of the tryptophan residues, 62, 63 or 108, the former two lying on the molecular surface and the latter at the entrance to the cleft of the hydrophobic box in the tertiary structure of lysozyme. The cationic head groups of the detergents CTAB and DTAB probably bind at the phenolic group of tyrosine 20 or 23 lying at the entrance to the cleft or at a side chain carboxyl group of Asp 101 or 103 on the molecular surface or Glu 35 which lies close to Trp 108 at the active site of the enzyme. For CPB cationic head group also, all the binding sites are essentially the

same as in the case of CTAB and DTAB, except for the tyrosine residue involvement. Since all the sites involved in the interactions lie in the non-helical region of the tertiary structure, a more ordered conformation is induced into the enzyme molecule, with the resultant increase in α-helical content.

Generally, when the polypeptide chain(s) of a globular protein folds in 3-dimensions to give a tertiary structure, all the polar groups will be exposed to the surface and the non-polar groups will be buried in the interior of the molecule so that the molecule is thermodynamically stable. In the case of lysozyme, which contains a larger proportion of non-polar amino acid residues, in addition to all the polar groups being on the surface, it is likely that some of the non-polar amino acid residues such as tryptophan, phenylalanine and few other non-polar residues lie on the molecular surface of the enzyme (Blake *et al.*, 1965). In such a situation, the hydrophobic interactions play a dominant role not only in maintaining the stability of the tertiary structure (Tanford, 1962) but also in the binding of detergents with longer hydrocarbon moieties involving several points of contact. Therefore, our binding studies on lysozyme with the cationic detergents reveal that hydrophobic interactions play a major role, while electrostatic interactions play only a minor role.

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Purification and characterization of putrescine synthase from cucumber seedlings. A multifunctional enzyme involved in putrescine biosynthesis†

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Abstract. The multifunctional enzyme, putrescine synthase has been purified from *Cucumis sativus* and characterized. This enzyme harbours agmatine iminohydrolase, ornithine transcarbamylase, putrescine transcarbamylase and carbamate kinase activities, whose concerted action results in agmatine → putrescine conversion. The enzyme resolved into two aggregation forms, enzyme aggregated and enzyme monomer upon electrophoresis at pH 8·3. Evidence has been provided by two-dimensional gel electrophoresis that both enzyme aggregated and enzyme monomer comprise of identical polypeptide chains. Under non-reducing conditions on sodium dodecyl sulphate–polyacrylamide gel electrophoresis, the protein moves as a single 150 KDa polypeptide; however, in the presence of 2-mercaptoethanol on sodium dodecyl sulphate–polyacrylamide gel electrophoresis, it migrates as 3 polypeptides of molecular weight 48,000, 44,000 and 15,000. The enzyme undergoes age-dependent *in vivo* proteolytic degradation from a 66 KDa polypeptide (primary translational product), through 48 KDa polypeptide to 44 KDa species and finally to small molecular weight peptides.

Keywords. Multifunctional enzyme; purification; proteolysis; putrescine biosynthesis.

Introduction

Although both higher plants and micro-organisms harbour the arginine decarboxylase pathway leading to putrescine biosynthesis, they employ entirely different sets of enzymic reactions distal to arginine decarboxylase (Adiga and Prasad, 1985; Tabor and Tabor, 1984). In micro-organisms, agmatine is cleaved to putrescine by agmatine ureohydrolase, in a reaction analogous to that of arginase (Tabor and Tabor, 1972; Morris and Pardee, 1966), whereas in plants, putrescine was presumed to be derived from a two-step hydrolysis of agmatine, catalysed by agmatine iminohydrolase and N-carbamyl putrescine amidohydrolase (Smith, 1963, 1965, 1970, 1971; Smith and Garraway, 1964). As against the above scheme of conversion of agmatine to putrescine proposed in plants, our recent work with

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Abbreviations used: PAGE, Polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G; BSA, bovine serum albumin; EA, enzyme aggregated; EM, enzyme monomer; CH-Sepharose, Carboxyhexyl-Sepharose; M_r, molecular weight; PMSF, phenyl methyl sulphonyl fluoride; DTT, dithiothreitol.

Lathyrus sativus seedlings has implicated the involvement of a versatile polycephalic enzyme *viz.*, 'putrescine synthase'* catalyzing these reactions. It was shown that due to the concerted action of its component enzymic activities (*viz.*, agmatine iminohydrolase, ornithine transcarbamylase, putrescine transcarbamylase and carbamate kinase), putrescine synthase converts agmatine to putrescine. The scheme of reactions catalyzed by this enzyme not only explains the non-accumulation of N-carbamyl putrescine in the plant but also envisages the conservation of labile carbamyl moiety in terms of cellular economy (Srivenugopal and Adiga, 1981, 1983a). During these studies, preliminary evidence was adduced for the operation of a similar sequence of reactions leading to putrescine elaboration in other plant systems including cucumber seedlings. However, it was not explored whether there is any discernible difference in terms of molecular characteristics of putrescine synthase from different higher plant systems. To investigate this possibility the purification and characterization of putrescine synthase from *Cucumis sativus* seedlings was undertaken and this paper deals with these aspects as well as the details of the reactions catalyzed by the purified enzyme protein.

Materials and methods

Materials

The following chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri, USA: micrococcal nuclease, Triton X-100, creatine phosphokinase, creatine phosphate, GTP and oligo(dT) cellulose. [^{14}C]-Urea (Sp. activity 38 mCi/m mol) and $H_2^{35}SO_4$ (carrier-free) were procured from Bhabha Atomic Research Centre, Bombay. Sources of other chemicals and cucumber seeds were same as referred to earlier (Srivenugopal and Adiga, 1981).

Since significant amounts of impurities present in commercial carbamyl phosphate interfered with the colorimetric assay employed, carbamyl phosphate was purified as described earlier (Srivenugopal and Adiga, 1981). ADP obtained commercially also harboured detectable amounts of ATP which interfered with the kinase assay (Lamprecht and Trautschold, 1974). The contaminant ATP was converted to ADP by treating with hexokinase prior to purification by chromatography on a. Dowex-1 column (Cohen and Carter, 1950).

Both radioactive (Sp. activity 30 μCi/m mol) and non-radioactive N-carbamyl putrescine were synthesized and purified according to the procedure detailed elsewhere (Srivenugopal and Adiga, 1980, 1983b). [^{35}S]-Methionine was prepared from *Escherichia coli* strain B grown on neutralized carrier-free $H_2^{35}SO_4$ according to the method of Crawford and Gesteland (1973).

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* Multifunctional enzyme with constituent activities of agmatine iminohydrolase (EC 3·5·3·12), putrescine transcarbamylase (EC 2·1·3·6), ornithine transcarbamylase (EC 2·1·3·3) and carbamate kinase (EC 2·7·2·2).

Methods

Putrescine carboxyhexyl-Sepharose for affinity chromatography: Putrescine carboxyhexyl-Sepharose (CH-Sepharose) was prepared as described earlier (Srivenugopal and Adiga, 1981).

Gel electrophoresis: Polyacrylamide gel electrophoresis (PAGE) under non-denaturing and denaturing conditions was carried out as detailed elsewhere (Davis, 1964; Laemmli, 1970).

Preparative electrophoresis: Preparative electrophoresis at pH 8·3, was carried out on 5% acrylamide slab gels (Davis, 1964). The regions of interest on the unstained slab gel were cut into small pieces and placed in glass tubes (1×13 cm) which had polymerized 3% acrylamide as the base. The bottoms of the tubes containing the polymerized gel were tied with dialysis tubing and electrophoresed for 18–24 h at 4°C. The electroeluted protein, collected in dialysis tubings was concentrated over sucrose and dialyzed against 10 mM Tris-HCl, pH 7·6 containing 0·5 mM Mn^{2+} and 2 mM 2-mercaptoethanol*.

Two-dimensional gel electrophoresis: Two-dimensional gel electrophoresis was carried out at pH 8·3 in the first dimension and sodium dodecyl sulphate (SDS)-PAGE as the second dimension. First dimension electrophoresis was carried out on 5% acrylamide gels, either in tubes or on slabs. A parallel tube/slot, which contained tracking dye was stained with the instant stain. The equilibrated gel was placed over the second dimension gel and sealed with 0·8% agarose and electrophoresis was carried out as described.

Immunological techniques

Preparation of antiserum: Albino rabbits (2 kg body wt.) were administered subcutaneously once a week with 100 μg of the purified enzyme protein in Tris-buffered saline, emulsified with equal volume of Freund's complete adjuvant at multiple sites. After 3 injections, a booster dose of 1 mg protein in saline was administered. After 6 days, animals were bled through the ear vein, serum separated and stored frozen. The immunoglobulin G (IgG) fraction from the antisera was prepared according to Cambell *et al.* (1970). Ouchterlony double immunodiffusion and immunoelectrophoresis were performed as described (Ouchterlony, 1967).

Immunoprecipitation: Known amounts of isolated enzyme protein and the IgG from the antisera to the enzyme (specific) or the antiserum raised to bovine serum albumin (BSA) were added to tubes containing the immunoprecipitation buffer (Tris-HCl buffer, pH 7·2, 0·1 M; 0·1 M NaCl; 0·5% Triton X-100) and incubated at 37°C for 1 h followed by standing at 4° C for 24 h. The immunoprecipitates were collected by centrifugation and washed 4 times with the above buffer. The final washing was carried out with the same buffer but without the detergent. When

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* Unless otherwise stated from now onwards 10 mM Tris-HCl, pH 7·6 containing 0·5 M Mn^{2+} and 2 mM 2-mercaptoethanol is referred to only as buffer.

radioactive antigens were to be immunoprecipitated, the washing buffer also contained the corresponding non-radioactive amino acid to minimize non-specific trapping of labelled protein.

Enzyme assays: The different component activities of the multifunctional enzyme, putrescine synthase, *viz.*, agmatine iminohydrolase, ornithine carbamylase, putrescine transcarbamylase and carbamate kinase and the overall reactions linked to either ornithine carbamyl transferase or carbamate kinase were assayed essentially as described elsewhere (Srivenugopal and Adiga, 1981). Unless otherwise stated, all the enzyme assays were conducted at 37°C for 2 h; carbamyl transferases were quantitated at 25°C for 30 min.

Definition of unit activity: Unless otherwise stated one unit activity is defined as the amount of enzyme required to produce 1 μmol of product (NH$_3$, N-carbamyl putrescine, citrulline or ATP)/h under standard assay conditions. Specific activity is expressed in units/mg of protein.

Other methods: Identification of amine products by paper chromatography was accomplished according to Srivenugopal and Adiga (1981). Protein was estimated according to Lowry *et al.* (1951) using crystalline BSA as a standard.

RNA was extracted from 6-day old cucumber seedlings (Palmiter, 1974) and poly (A)$^+$ RNA was separated from the total RNA on oligo(dt)-cellulose (Aviv and Leder, 1972). *In vitro* translation of isolated poly (A)$^+$ RNA was carried out in rabbit reticulocyte lysates according to Ravishankar and Padmanaban (1983). Homologous translation using a supernatant fraction from S$_{27}$ homogenates after centrifugation at 4°C at 27,000 g of 6-day old cucumber cotyledons was performed according to the protocol adopted by Srivatsan and Padayatty (1976).

Purification of cucumber putrescine synthase

In order to determine the particular developmental stage of the seedlings at which the putative putrescine synthase can be isolated with satisfactory yields, the enzyme was extracted at different stages of the plant development. The component activities were estimated in the crude enzyme extracts (see below). The results (figure 1) clearly indicated that all the enzyme activities peak at day 6 of seedling growth and slowly decline thereafter. In view of these observations 6-day old seedlings were employed as the source for enzyme purification.

Thoroughly washed plant tissue was homogenized in a precooled Waring blender or by grinding in a glass pestle and mortar with glass powder in the presence of 20 mM Tris-HCl buffer, pH 8·5 containing 5 mM 2-mercaptoethanol and 0·5 mM MnCl$_2$ and centrifuged at 15,000 g for 15 min. Unless otherwise stated all the operations were carried out at 4°C. When used for activity assays the clear supernatant was dialyzed against 10 mM Tris-HCl, pH 7·5 containing 2 mM 2-mercaptoethanol and 0·5 mM Mn^{2+} and the clarified protein fraction was employed for further purification. The crude extract was adjusted to 7·5 mM MnCl$_2$ concentration and stirred for 60 min. Precipitated nucleoproteins were removed by centrifugation.

The clarified MnCl$_2$-supernatant was brought to 90% saturation with ammonium sulphate and stirred for 1 h to complete precipitation. The precipitate was collected by centrifugation and dissolved in a minimum volume of 50 mM Tris-HCl buffer,
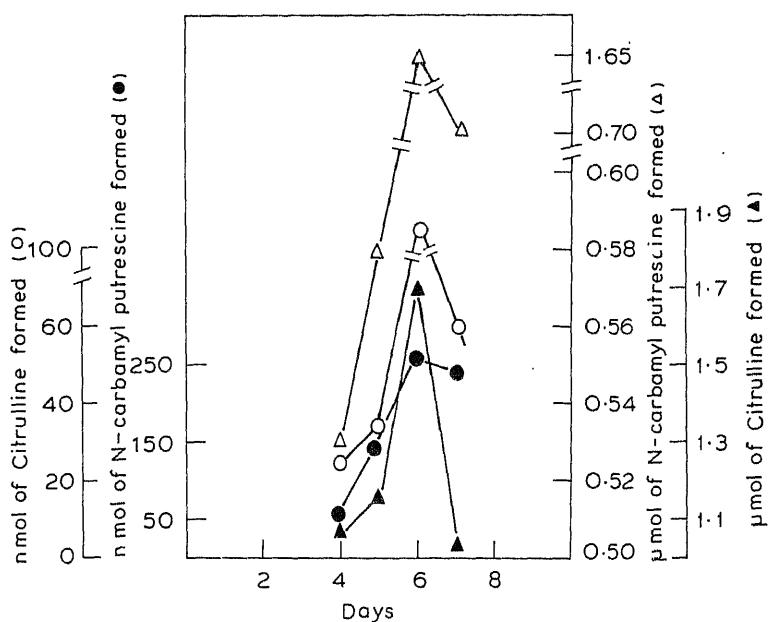
Figure 1. Changes in the component activities of putrescine synthase during the development of cucumber seedlings.

Cucumber seedlings were harvested at various stages of growth and crude enzyme extracts were prepared as described in 'methods'. Agmatine iminohydrolase, putrescine transcarbamylase, ornithine transcarbamylase and the complete reaction (agmatine + ornithine $\xrightarrow{P_i}$ citrulline + putrescine + NH_3) were measured.

($\blacktriangle$), Ornithine transcarbamylase; ($\triangle$), putrescine transcarbamylase; ($\bullet$), agmatine iminohydrolase; ($\bigcirc$), complete reaction.

pH 8·0 containing 2 mM 2-mercaptoethanol and 0·5 mM Mn^{2+} and desalted on a Sephadex G-25 (2×75 cm; coarse 100–300 μ) column pre-equilibrated with buffer. The salt-free ammonium sulphate precipitated fraction was applied onto a column of putrescine–CH-Sepharose (10 ml bed volume; $1·5 \times 7·5$ cm) pre-equilibrated with buffer and recycled. The column was washed extensively with buffer to remove the unbound proteins and elution of the column with 5 mM putrescine resulted in dislodging of the bound protein as a single peak (data not shown). The dialysed, pooled protein fraction, when assayed harboured all the constituent activities of putrescine synthase and gave positive results when tested for the overall reactions (*viz.*, agmatine + ornithine → putrescine + citrulline + ammonia). At this stage, the protein essentially resolved into two bands, enzyme aggregated (EA) and enzyme monomer (EM) on alkaline PAGE (figure 2a). These two bands were not separable by other techniques like gel filtration on Sephadex G-200 or ion-exchange chromatography on DEAE-cellulose.
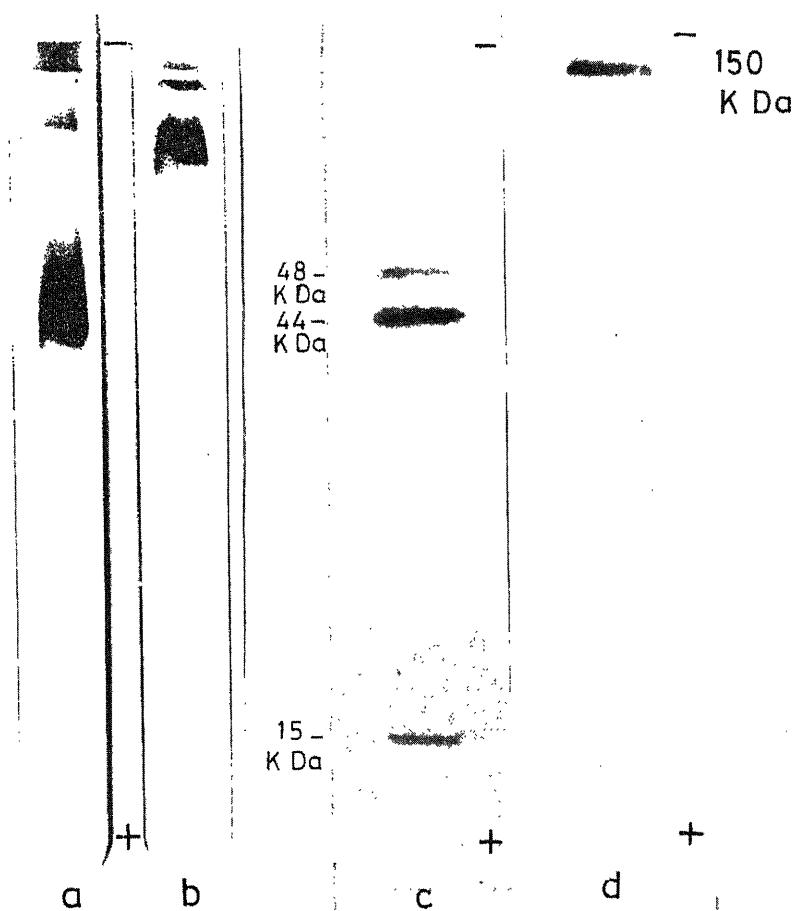
Figure 2. PAGE of putrescine Sepharose eluate.
 (a, b), Electrophoresis at pH 8·3 under non-denaturing conditions for 6 h and 3 h, respectively on 7·5% acrylamide gels. (c, d), Electrophoresed in the presence of SDS under reducing and non-reducing conditions respectively on 10% acrylamide gels. Protein load of about 150 μg was employed in each case.

Results

Purification of the enzyme

Putrescine synthase from the cucumber seedlings was purified by affinity chromatography on putrescine CH-Sepharose, the procedure developed for purification of *L. sativus* enzyme (Srivenugopal and Adiga, 1981). However, the recovery of the enzyme activities was relatively less satisfactory (tables 1 and 2). The expected constant ratios of specific activities of constituent enzyme activities during different stages of purification as a criterion for association of different enzyme activities with a single protein, could not be demonstrated because of uncontrolled proteolytic degradation, the existence of more than one catalytically

Table 1. Summary of purification of putrescine synthase from *C. sativus*.

Purification step	Specific activity (μmol product/mg protein/h assay)				Total activity (specific activity $\times$ total protein (mg))			
	Agmatine imino-hydrolase[a]	Ornithine transcarba-mylase	Putrescine transcarba-mylase (N-Carbamyl putrescine synthesis)	Agmatine + Ornithine → Citrulline + Putrescine + NH_3	Agmatine imino-hydrolase	Ornithine transcarba-mylase	Putrescine transcarba-mylase (N-Carbamyl putrescine synthase)	Agmatine + Ornithine → Citrulline + Putrescine
Crude extract	0·023	0·22	0·131	0·01	192·0	177·2	1055·0	67·0
Ammonium sulphate precipitation	0·18	0·62	0·17	0·02	71·6	247·2	294·8	7·9
Affinity chromatography[b]	2·64 (110·7)[c]	4·0 (14·36)[c]	4·0 (31·6)[c]	1·06 (127)[c]	4·8 (2·5)[d]	5·9 (0·33)[d]	7·6 (0·72)[d]	1·98 (2·9)[d]

[a] Agmatine iminohydrolase was assayed by N-carbamyl putrescine isolation method. [b] On putrescine-CH-Sepharose column. [c] Fold purification. [d] Per cent recovery.

Table 2. Purification of carbamate kinase (component of multifunctional enzyme, putrescine synthase) from cucumber seedlings.

Purification step	Specific activity units (μmol of ATP produced/mg protein/h)		
	Carbamyl phosphate + ADP + $P_i \rightarrow$ ATP + NH$_3$ + CO$_2$	N-Carbamyl putrescine + ADP + P_i $\rightarrow$ ATP + Putrescine + NH$_3$ + CO$_2$	Agmatine + ADP + $P_i \rightarrow$ ATP + Putrescine + 2NH$_3$ + CO$_2$
Crude extract	0·049	0·05	0·028
MnCl$_2$ supernatant	0·05	0·05	0·03
Ammonium sulphate precipitation	0·474	0·03	0·129
DEAE-cellulose	0·143	0·079	0·179
Affinity eluate	—	—	0·523

active molecular species and the labile nature of the constituent enzyme activities as purification progressed. Estimation of NH$_3$ for assaying agmatine iminohydrolase activity during the early steps of purification also proved unsatisfactory because of the presence of 3 types of amine oxidases in this plant system, all utilizing agmatine as substrate (Percival and Purves, 1974) (data not given). Chromatography on DEAE-cellulose was found necessary as an additional purification step prior to affinity chromatography to enhance the efficacy of the affinity matrix. The final purification protocol employed was in brief as follows: the protein from ammonium sulphate precipitation step was loaded onto a DEAE-cellulose column pre-equilibrated with the buffer; this was followed by sequential washing of the column with the buffer and 0·1 M KCl, and subsequent elution of the adsorbed protein with 0·3 M KCl. The resultant enzyme preparation exhibited all the component enzyme activities of putrescine synthase described earlier (Srivenugopal and Adiga, 1981). The protein was concentrated by ammonium sulphate precipitation and desalted by exclusion on Sephadex G-25 column. This concentrated DEAE-cellulose eluted protein was finally applied onto putrescine CH-Sepharose used as a column.

Molecular characteristics of the enzyme

On electrophoresis at pH 8·3, putrescine-Sepharose eluate was resolved into two protein bands (EA and EM) on both 7·5% or 5% polyacrylamide gels. No additional bands could be detected even when the protein as high as 150–200 μg was loaded to detect possible presence of other minor species. Only when protein load was large (200 μg) a characteristic stainable streak was observed (figure 2a,b). Results of two-dimensional gel electrophoresis show that the two molecular entities resolved on electrophoresis represent the same protein, but with different degrees of aggregation.

Gel filtration

When the affinity purified preparation was loaded onto a Biogel-P-300 column, the protein was eluted in the void volume. Agmatine iminohydrolase activity of the

excluded protein (assayed as a representative of component activities of putrescine synthase) was comparable to that of the putrescine CH-Sepharose eluate on unit protein basis (data not shown). The stainable protein pattern resolved on the gel during SDS-PAGE was also identical in both cases. It would therefore appear that both 48 KDa and 44 KDa polypeptides along with the minor degradation products exist as a large molecular entity which could not be resolved into the components under milder conditions of gel filtration.

SDS-PAGE

When either the putrescine-Sepharose eluate or the two constituent molecular species separated on PAGE was individually subjected to SDS-PAGE under non-reducing conditions, a single stainable protein band with a molecular weight (M_r) of 150,000 was observed (figure 2d). However, in the presence of 2% (v/v) 2-mercaptoethanol, two well defined bands *viz.*, 48 KDa and 44 KDa polypeptides besides other fast moving small M_r peptides (figure 2c) were observed in each of these cases. The small M_r peptides observed presumably represent proteolytic degradation products of the protein either *in vivo* or during purification. However, the appearance of the two bands of higher M_r *viz.*, 48,000 and 44,000 with almost equal intensity is not easily explained at this stage and may represent proteolytic degradation products of a single protein species or two different types of subunits held together by disulphide bonds.

Relationship between the two enzyme forms

Conclusive evidence that these two molecular species (*i.e.*, EA and EM) represent different forms of the same basic unit, stems from analysis by two-dimensional gel electrophoresis. The protein separated as EA and EM components on alkaline gels was subjected to SDS-PAGE in the second dimension (figure 3). Both EA and EM exhibited identical pattern on gels since both of them consisted predominantly of 48 KDa and 44 KDa species. Efforts to minimize the appearance of the multifunctional protein in the two aggregated states by adding detergents like Triton X-100 during purification proved futile.

Evidences for proteolytic degradation

As indicated earlier, the multiple protein bands observed in SDS-PAGE presumably reflect proteolytic degradation of the enzyme. In an attempt to prevent proteolysis, phenyl methyl sulphonyl fluoride (PMSF) at 0·1 and 1·0 mM concentrations was included in the buffers during tissue homogenization as well as subsequent steps; but this could not curtail the appearance of the enzyme protein as 48 KDa and 44 KDa species on SDS-PAGE to any discernible extent indicating that this phenomenon represents an *in vivo* situation. Supporting evidence for the above arises from the observation that the enzyme protein prepared from the seedlings at different stages of development showed variable relative proportions (as assessed from their staining intensity) of 48 KDa and 44 KDa species on SDS-PAGE in the presence of 2-mercaptoethanol, despite the fact that under non-reducing conditions, the protein uniformly exhibited M_r of 150,000. That the age-dependent *in vivo* proteolysis was responsible for these observations is

Figure 4. Isoelectric focusing and two dimensional gel electrophoresis of fish brain tubulins. (a), Isoelectric focusing was performed as described in experimental section with 40 μg of fish and goat brain tubulin purified by two cycles of assembly and disassembly. (b), Electrophoresis in the second dimension of duplicate unstained IEF gel of catfish brain tubulin, obtained as in (a) was performed in 7·5% acrylamide-SDS gel. Staining was done in Coomassie blue.

(Langford, 1978), yeast (Kilmartin, 1981) or Ehrlich ascites cells (Doenges *et al.*, 1979) also self-assemble in the absence of known high M_r MAPs. The significance of low M_r proteins which appear in trace amounts after first cycle of assembly is not clear as they disappear after subsequent assembly cycles showing that they do not represent an absolutely necessary factor for assembly of fish brain tubulin. Low M_r proteins have been reported in oocytes of sea urchin and surf clam (Suprenant and Rebhun, 1981, 1984) and flagellar tubulin of sea urchin (Binder and Rosenbaum, 1978), some of which may be related to the low M_r MAPs (M_r 30,000–35,000) present in MT proteins isolated from bovine brain (Berkowitz *et al.*, 1977). Alternatively, they may represent MT binding, assembly promoting polypeptides similar to the fragments of MAP-2 (M_r 32,000–39,000), produced by proteolytic cleavage (Vallee, 1980). Further work is necessary to determine what role, if any, the low M_r proteins play in the regulation of fish tubulin assembly.

Figure 5. Proteolytic digestion patterns of α and β tubulin subunits from catfish and goat brain. Catfish and goat brain tubulin α and β subunits were separated on a 7·5% SDS-polyacrylamide gel and the cut out bands containing 10 μg protein were digested in a 5% stacking gel with 0·25 μg of *S. aureus* V8 protease and the resulting peptides were separated in a 15% SDS-polyacrylamide gel according to Cleveland *et al.* (1977). (a), (b) Catfish and goat brain α tubulins, and (c), (d) catfish and goat brain β tubulins, respectively.

he assembly of fish tubulin at low temperature shows it to be more stable than nmalian brain tubulin and the temperature of isolation (either 18–20°C or 37°C) s not significantly alter the yield. Similar temperature stability has been rted in case of dog fish brain tubulin (Langford, 1978), invertebrate ilothermic animals like echinoderms and molluscs (Suprenant and Rebhun, ., 1984).

oelectric focusing and two dimensional gel electrophoresis resolves catfish lin into several isoforms (figure 4). These variants are different from goat brain lin isoforms. Microheterogeneity of tubulin from several organisms have been rved and is generally indicative of tubulin multigene families (Alexandraki and erman, 1981; Mischke and Pardue, 1982; Hall *et al.*, 1983; Lopata *et al.*, 1983).

Figure 5. *In vitro* translation of cucumber putrescine synthase.

(a), The RNA isolated from 6-day old cucumber seedlings was translated in the reticulocyte lysate system and immunoprecipitated with IgG fraction of the antiserum raised against purified putrescine synthase and immunoprecipitates were analyzed on 7·5% SDS-PAGE and monitored by fluorography. Lane (i) total RNA 5 μg and (ii) 2 μg of poly (A$^+$)RNA.

(b), Cucumber cotyledon extracts active in protein synthesis (S_{27} fraction) were prepared as described in 'materials and methods'. Cell-free synthesis of the enzyme was carried out by using endogenous messengers, immunoprecipitated and analyzed as described above. Lane (i) immunoprecipitated using specific IgG and (ii) immunoprecipitated using IgG prepared from antiserum to bovine serum albumin.

Cell-free synthesis of putrescine synthase using homologous translation system

In order to examine the nature of primary translation product synthesized in the homologous cell-free translation system with all the enzymatic machinery required to process putrescine synthase, the active extracts from the cucumber cotyledons

capable of supporting [^{35}S]-methionine incorporation into the protein were prepared as described and translation carried out using endogenous mRNA. Upon analyzing the immunoprecipitated proteins on SDS-PAGE it became evident, that in addition to the expected protein species of 66 KDa, significant amount of 48 KDa species were detectable (figure 5b). This clearly supported the earlier finding that proteolytic processing of the putrescine synthase complex is inherent to the cucumber seedlings (figure 5b).

Evidence for proteolysis from immunological techniques

When the purified IgG from the specific antiserum raised against the putrescine-CH-Sepharose eluate was allowed to cross-react with the multifunctional protein during different stages of purification, a single precipitin line was observed both during double immunodiffusion and immunoelectrophoresis (figure 6a,b). This
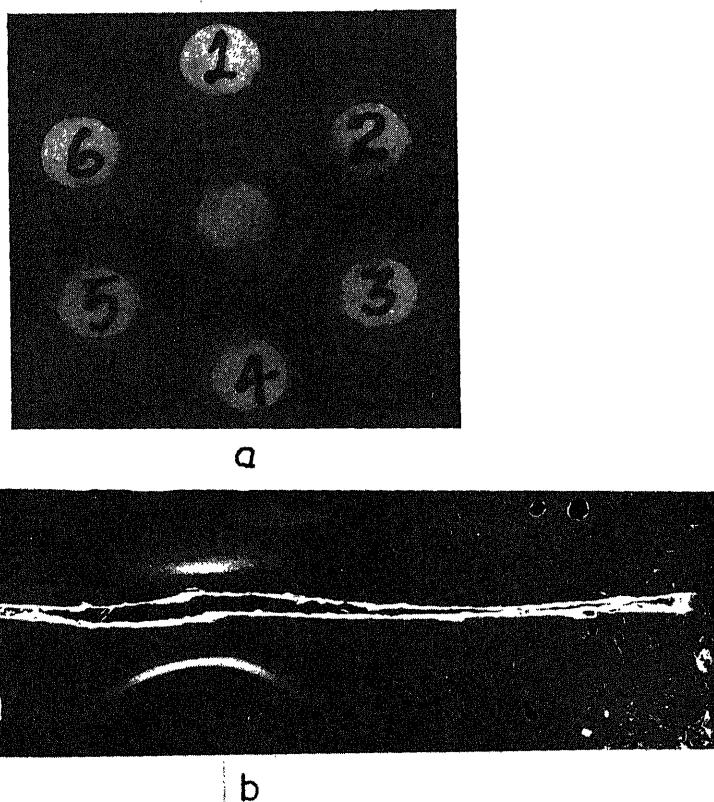
Figure 6. Ouchterlony immunodouble diffusion.

The centre well contained IgG prepared from the antiserum raised against putrescine Sepharose eluate. Outer wells contained the protein fractions from (1) crude extracts, (2) MnCl$_2$ supernatant, (3) DEAE-cellulose eluate, (4) and (6) putrescine Sepharose eluate and (5) BSA.

Immunoelectrophoresis: The centre trough contained specific IgG for putrescine synthase. The well contained (i) crude extracts of cucumber seedlings and (ii) putrescine Sepharose eluate.

clearly attests to both the specificity of the antibody and the immunochemical purity of the isolated putrescine synthase.

To ascertain the purification step at which the maximum proteolysis of the enzyme protein becomes evident, putrescine synthase was immunoprecipitated at different stages of the purification and analyzed by SDS-PAGE. Proteolytic degradation of the protein becomes discernible in crude extracts itself and throughout the purification protocol employed, this same degradation pattern more or less persists (data not shown).

General properties

Putrescine synthase was highly unstable since all the component activities as well as complete reactions seemed to rapidly decrease as purification progressed. Owing to the presence of more than one species of the enzyme at any stage of purification constant ratios of the component activities at different stages of purification could not be obtained. On the premise that such small M_r degradation products may be inhibitory to the catalytic activity(ies), further purification of the two enzyme species EA and EM by preparative electrophoresis and the individual constituent enzyme activities were analyzed; this led to dramatic improvement in the catalytic efficiencies of all the activities associated with both EA and EM; EA displayed higher specific activity on unit $A_{280\ nm}$ basis for all the component activities as well as of the complete reaction, compared to EM (table 3). The reaction *viz.*, N-carbamyl putrescine + ornithine → putrescine + citrulline + NH_3, which could not readily be quantified in the affinity eluate or resolved EM, was clearly assayable with EA. Agmatine iminohydrolase activity was 3 times higher in EA than in EM, on unit protein basis. Further characterization of these component activities or the overall reactions were severely hampered by the poor yields and instability of the enzyme preparations. For example, the carbamate kinase activity which could be unequivocally demonstrated upto DEAE-cellulose chromatography step could not reproducibly be assayed individually after affinity chromatography. However, its

Table 3. Component activities of putrescine synthase in EA, EM and putrescine-Sepharose eluate.

Enzyme Source	Agmatine iminohydrolase		Ornithine trans- carbamylase	Putrescine transcarba- mylase (N-Carbamyl- putrescine synthase)	Agmatine[a] Ornithine → Citrulline + Putrescine + NH_3	N-Carbamyl putrescine + Ornithine[a] → Citrulline + Putrescine
	NH_3	N-Carbamyl putrescine synthesis				
Putrescine- Sepharose eluate	0·687	0·7	0·436	3·8	0·218	Not detectable
EA	3·0	3·1	4·6	10·4	1·25	1·43
EM	1·3	1·2	3·9	5·9	0·072	0·072

Activities expressed as μmol of product formed/mg/h.

[a] Citrulline was quantitated in the complete reactions linked to ornithine transcarbamylase.

participation in the overall reaction linked to carbamate kinase (*viz.*, agmatine $+ ADP + P_i \rightarrow ATP + putrescine + 2NH_3 + CO_2$) could be convincingly demonstrated. The other two reactions *viz.*, carbamyl phosphate $+ ADP \rightarrow ATP + NH_3 + CO_2$ and $NCP + ADP + P_i \rightarrow putrescine + ATP + NH_3 + CO_2$ could be followed upto DEAE-cellulose chromatography (table 2).

The component enzyme activities

Agmatine iminohydrolase activity and the arsenolytic cleavage reaction ($NCP \rightarrow$ putrescine) share similar properties as those exhibited by the *L. sativus* enzyme (data not given). All the component activities as well as complete reactions had comparable pH optima to the enzyme activities catalyzed by *L. sativus* enzyme (data not given).

The carbamyl transferases: Ornithine transcarbamylase was the most stable activity among those harboured by the multifunctional enzyme. Both ornithine and putrescine transcarbamylase (N-carbamyl putrescine synthesis) depended on added DTT for their activity (table 4). Mn^{2+} or Mg^{2+} (1 mM) stimulated ornithine transcarbamylase but inhibited putrescine transcarbamylase activity.

Table 4. Comparison of carbamyltransferase activities of putrescine synthase from cucumber seedlings.

	Activity units/mg protein	
Assay conditions	Ornithine transcarbamylase (citrulline)	Putrescine transcarbamylase (N-Carbamyl putrescine)
Control	0·5	1·59
− DTT	0·36	0·88
+ Mg^{2+} (1 mM)	1·46	0·47
+ Mn^{2+} (1 mM)	1·41	0·94

Citrulline and N-carbamyl putrescine were quantitated for measuring ornithine transcarbamylase and putrescine transcarbamylase (biosynthetic direction) activities, respectively.

Overall reaction linked to ornithine transcarbamylase: The products of complete reaction linked to ornithine transcarbamylase, *viz.*, putrescine and citrulline have been identified by paper chromatography. Unlike the component carbamyl transferase activities, the complete reaction exhibits an absolute requirement for dithiothreitol (DTT) (table 5). In the absence of P_i and Mg^{2+}, very little of citrulline is produced as measured in ammonia eluates of Dowex-column.

Discussion

The data presented above clearly support the conclusion that the polycephalic putrescine synthase does function in cucumber system also, notwithstanding the greater complexity of its structural and functional organization *vis-a-vis* its

Table 5. Requirements for the complete reaction coupled to ornithine transcarbamylase activity of putrescine synthase from cucumber seedings.

Assay conditions	Activity units (nmols of citrulline formed/2 h assay)/mg protein
Complete system	19·0
− DTT	2·0
− Ornithine	3·7
− Agmatine	Not detectable
− P_i	7·4
− Mg^{2+}	6·0
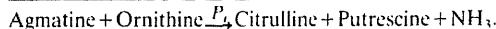
Agmatine + Ornithine $\xrightarrow{P_i}$ Citrulline + Putrescine + NH_3.
The complete assay mixture (1·0 ml) consisted of 100 μmol of Tris-HCl (pH 8·5), 2 mM DTT, 5 mM Mg^{2+}, 5 mM $Na_2 HPO_4$, 2·5 μmol agmatine and 5 μmol of ornithine. Assay was initiated by the addition of 200 μg of pure enzyme. Citrulline was separated from agmatine by elution with NH_4OH from a Dowex 50-H^+ column as described by Srivenugopal and Adiga (1981).

counterpart in *L. sativus.* The first indication that all the expected component activities are associated in a close structural and functional organization in this plant system also, stems from the coordinate changes in their individual catalytic activities during different stages of seedling growth (figure 1). Corroborative evidence for such intimate association is provided by the copurification of all the component activities as well as those mediating the overall reactions, during affinity chromatography on putrescine-CH-Sepharose column (tables 1 and 2). Among the major constraints towards further detailed characterization, the extreme lability and susceptibility of the enzyme to proteolysis dominated. Such impediments, however, were not totally unexpected since similar difficulties were encountered earlier with many other multifunctional proteins (Schweizer, 1980; Lampkin IV· *et al.*, 1976; Gaertner and Cole, 1977; Coleman *et al.*, 1979). Additionally, the finding that the enzyme exists as two separable molecular entities EA and EM, with differing degree of catalytic efficiency added another dimension to the complexity (figure 2a, b; table 5). Although this initially raised the discrete possibility that the two molecular forms (EA and EM) are either isolation artifacts or represent isoenzymes contributed by different plant tissues (embryoaxes and cotyledons), further investigations revealed that they, indeed, represent different aggregated states of the same basic enzyme unit of M_r 150,000. While the exact relationship between the two molecular forms is still enigmatic, lower catalytic efficiency coupled with relative abundance of smaller sized constituent polypeptides of EM provide presumptive evidence for a precursor–product relationship between them. Another noteworthy feature of these constituent polypeptides is that they themselves seem to arise as the result of limited proteolytic modification of a larger biosynthetic unit (66 KDa species) of the enzyme. This is based on the observation that on translation of isolated mRNA from cucumber seedlings in a heterologous cell-free system (derived from rabbit reticulocyte lysates), immunoprecipitable radioactivity is associated with a single polypeptide chain of apparent M_r of 66,000 (figure 5a). Similarly, such a biosynthetic precursor could be detected as the primary translational product in the homologous translation system prepared from

6-day seedlings along with other smaller M_r components characteristic of putrescine synthase enzyme as expected (figure 5b). The fact that the 66 KDa protein is not detectable in putrescine synthase purified from day-6 seedlings when peak activity was encountered (figures 1 and 4) raises the possibility that limited proteolysis may also be involved in the 'maturation' of enzyme to exhibit the maximum catalytic efficiency.

It is noteworthy that the age-dependent *in vivo* proteolytic modification of cucumber putrescine synthase has its counterparts in several of the multifunctional enzyme systems investigated hitherto. The classical examples of this category of enzyme are the 'arom' conjugate of *N. crassa* (Welch and Gaertner, 1980) and tryptophan synthase of yeast (Holzer *et al.*, 1973; Crawford, 1980). Other examples for specific age-dependent proteolytic degradation of proteins are carbamyl phosphate synthase-aspartate transcarbamylase-dihydroorotate dehydratase of *Drosophila* (Azou *et al.*, 1981) and cAMP dependent protein kinase of mouse (Beer *et al.*, 1984). While the physiological significance of the age-dependent proteolysis of cucumber putrescine synthase is currently unclear, such specific degradation has been implicated in altered structural organization of chimeric enzymes leading to modified metabolic chanelling of reactants (Welch and Gaertner, 1980). As demonstrated with putrescine synthase in the present study, investigations on the 'arom' conjugate of *N. crassa* have shown that even when the enzyme protein suffers multiple proteolytic clips, the resultant polypeptides are still tenaciously held to retain the total catalytic efficiency and move as a single entity on alkaline-PAGE; only under severe denaturing conditions, they could be resolved into multiple bands. The analogy between the fungal enzyme and the plant putrescine synthase is obvious from the data presented.

In view of the complex pattern of constituent polypeptides of the cucumber putrescine synthase as observed on SDS-PAGE, an alternate possibility was considered that the enzyme might be harboring its associated activities more as a multi-enzyme complex rather than a single multifunctional entity. However, the nature of its primary translational product in the heterologous cell-free translational system together with age-dependent pattern of its constituent polypeptides do not seem to favour the above proposition.

Notwithstanding this greater complexity in terms of structural organization *vis-a-vis L. sativus* putrescine synthase (Srivenugopal and Adiga, 1981), the cucumber enzyme besides mediating all the constituent enzyme activities, also seems to catalyze the phosphorylytic conversion of N-carbamyl putrescine to putrescine in a putrescine transcarbamylase-type of reaction. This is exemplified by the finding that (i) both N-carbamyl putrescine synthesis and arsenolysis are mediated by the enzyme, (ii) the complete reaction *viz.*, agmatine → putrescine conversion, coupled to ornithine transcarbamylase requires both Mg^{2+} and P_i; citrulline production is untraceable in the absence of either of the substrates, *viz.*, agmatine or ornithine, (iii) both the transcarbamylases as well as the complete reactions require a reducing agent for optimal catalysis, (iv) requirements for arsenolysis are similar to those observed with *L. sativus* enzyme, (v) pH optima of various component enzymes fall within a narrow range and (vi) both the coupling activities, *viz.*, ornithine transcarbamylase and carbamate kinase are demonstrable with agmatine as well as N-carbamyl putrescine as substrates. It is significant that in the absence of P_i, ornithine and ADP, the reaction does not progress beyond

N-carbamyl putrescine, presumably due to the thermodynamic constraint for the phosphorylytic cleavage of N-carbamyl putrescine by putrescine transcarbamylase (Roon and Barker, 1972). Of interest is the finding that the two coupled carbamyl transferases are differentially regulated by Mg^{2+} and Mn^{2+}, in that ornithine transcarbamylase is stimulated by both the co-factors whereas putrescine transcarbamylase is inhibited (table 4). The stimulatory effect of ornithine transcarbamylase is in line with the finding that the complete reaction linked to ornithine transcarbamylase requires Mg^{2+} (table 5).

From the foregoing, the overall picture that emerges clearly support the concept that agmatine cycle proposed for *L. sativus* is also operative in cucumber system and that the multifunctional putrescine synthase catalyzing agmatine → putrescine transformation channellizes N-carbamyl putrescine with resultant intact transfer of carbamyl group to support citrulline production thus conserving energy which otherwise would be dissipated.

Acknowledgement

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The mode of inhibition of the biosynthesis of phenylalanine ammonia lyase by its product cinnamic acid in aging potato parenchyma tissue

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Abstract. Excised potato parenchyma tissue upon aging in air and light, showed synthesis of RNA, as assessed by the incorporation of [^{3}H]-uridine, reaching a maximum in 18 h. The increase in RNA synthesis was in parallel with the increase in phenylalanine ammonia lyase synthesis. The treatment of the tissue, initially with actinomycin D at 15 μg/g tissue, or with trans-cinnamate at 3·5 mM, caused 65% and 50% inhibition of RNA synthesis, respectively. The inhibition was reduced to 20% by delaying the treatment of trans-cinnamate to the ninth hour. A comparative study on the nuclear fractions, isolated from trans-cinnamate treated and untreated aged parenchyma tissues showed that trans-cinnamate treatment inhibited the *in vitro* RNA synthesis by 38%. Studies on the *in vivo* synthesis of PAL and other proteins showed that trans-cinnamate treatment mainly impaired the *de novo* synthesis of PAL. The total RNA, isolated from trans-cinnamate treated parenchyma, was 66% less efficient in the translation of PAL, in cell free wheat germ protein synthesizing system. The translation product, purified by affinity chromatography on phenylalanine conjugated sepharose-4B was found to be homogeneous and showed a single radioactive peak corresponding to protein band on polyacrylamide gel electrophoresis.

Keywords. Potato parenchyma; induction of phenylalanine ammonia lyase; trans-cinnamate as inhibitor; RNA synthesis; transcriptional control.

Introduction

The modulation of the induction of phenylalanine ammonia lyase (PAL) (EC 4.3.1.5) by the metabolites of phenyl-propanoid pathway was reported in many cases (Hanson and Havir, 1981; Jones, 1984). In these studies it was suggested that these intermediates modulate the enzyme activity by a post translational mechanism rather than inhibition at transcriptional level. Thus the effect of trans-cinnamate could be at the processing or translation of PAL-mRNA rather than at the level of its synthesis (Lamb, 1979; Smith and Rubery, 1979.)

Our earlier studies showed that aging excised potato parenchyma tissue exhibited an essential requirement for oxygen for the development of PAL activity (Shirsat and Nair, 1976, 1981). The deprivation of oxygen in the incubation atmosphere caused accumulation of trans-cinnamate, much beyond the steady state level found in aerobically aged tissue, due to retardation of hydroxylation to *p*-coumaric and its

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Abbreviations used: PAL, Phenylalanine ammonia lyase, NRB, nuclear resuspension buffer; TCA, trichloroacetic acid; PAGE, Polyacrylamide gel electrophoresis.

further metabolism. As a consequence of this PAL induction was inhibited (Shirsat and Nair, 1981). The inhibition of PAL synthesis either by anaerobiosis or on exogeneous supply of trans-cinnamate was proved by determining the *in vivo* synthesis of PAL assessed by the increase in enzyme activity as well as by [^{14}C]-leucine incorporation into homogeneous PAL purified by affinity chromatography (Shirsat and Nair, 1981). These observations suggested that transcinnamate may exert its effect either at transcriptional level or translational level. The results presented in this paper will support the idea that trans-cinnamate affects PAL synthesis by transcriptional control.

Experimental

Parenchyma tissue slices from freshly harvested potatoes (*Solanum tuberosum* Linn) Kufri Chandramukhi cultivar, were used in these studies.

RNA synthesis

Freshly excised parenchyma tissue blocks weighing 5 g were dipped for 45 min in 2 ml solution of [^{3}H]-uridine (25 μCi) having a specific activity 9100 mCi/mmol (Isotope Group, Bhabha Atomic Research Centre, Bombay). The suspension was intermittently shaken to ensure uniform uptake of the label by the tissue. After 45 min the tissues were spread on a moist filter paper in a petri-dish and exposed to air and light for different time intervals upto 36 h. At the end of the desired period the tissues were washed well with water and taken for isolation of RNA after freezing the tissue in liquid nitrogen.

In all experiments precautions were taken to avoid bacterial contamination during incubation and RNA degradation, by sterilizing all glassware by dry heat (200°C, 8 h) and preparing all solutions in sterile distilled water.

RNA was isolated according to the method of Tao and Khan (1976) by phenol extraction procedure modifed by Nair and Ussuf (1978) for isolation from potato tissue. The RNA was precipitated with cold ethanol by keeping overnight at -30°C. The precipitate obtained by centrifugation was washed twice with 70% ethanol containing 1% potassium acetate. The precipitate was dissolved in 2 ml of 0·01 M Tris-HCl buffer pH 8·0, centrifuged and an aliquot of 0·05 ml was taken for counting in a Beckman liquid scintillation counter.

RNA content was determined by measuring the absorbance at 260 nm. One A_{260} unit corresponded to 50 μg of RNA.

Actinomycin D and trans-cinnamate treatment

The tissues after soaking in a solution of [^{3}H]-uridine as described above were then treated with either 75 μg of actinomycin D or 3·5 mM of trans-cinnamate solution separately. In these experiments the tissues were aged for 16 h in the presence of air and light. In another experiment treatment with trans-cinnamate was delayed by 2, 9 and 12 h and afterwards the tissues were incubated upto 16 h. RNA was isolated and radioactivity determined as described above.

Isolation of nuclei

The nuclei were isolated from fresh or aged parenchyma tissue according to the method of Luthe and Quartrano (1980), modified to suit for potato tissue.

The tissue slices were gently ground for 30 s in a precooled mortar and pestle with 2 vols of Honda buffer (Honda *et al.*, 1966) containing 0·44 M Sucrose, 2·5% (w/v) Ficoll (molecular weight 40,000) 5% (w/v) Dextran 40, 25 mM Tris-HCl (pH 7·6), 10 mM $MgCl_2$, 10 mM, β-mercaptoethanol and 0·5% (v/v) Triton X-100. The homogenate was diluted with 5 vols of buffer and filtered through 4 layers of cheese cloth. The filtrate was allowed to stay in ice for about 2 min for the starch particles to settle. The solution was decanted into a centrifuge tube without disturbing the settled starch and was centrifuged at 5850 *g* for 5 min and the supernatant was discarded. The nuclear pellet was then uniformly suspended in nuclear resuspension buffer (NRB), containing 50 mM Tris-HCl (pH 7·8), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol and 20% glycerol. The remaining starch particles were allowed to settle before the nuclear suspension was taken for sucrose gradient centrifugation. The discontinuous sucrose gradient consists of 5 ml layers of 0·5, 1·0, 2·0 and 3·0 M sucrose dissolved in a solution of 25 mM Tris buffer (pH 7·5) and 10 mM $MgCl_2$. The tubes were centrifuged at 4080 *g* in a Sorvall HB_4 swinging bucket rotor for 30 min. The nuclei banded between 1 and 2 M sucrose were removed by a Pasteur pippet and washed with Honda buffer and NRB and resuspended in NRB for further experiments.

DNA dependent RNA polymerase activity of nuclei

RNA polymerase was assayed as described by Kahl and Weohselberger (1977) with slight modification. The assay mixture consisted of 100 mM Tris-HCl pH 8·0, 2·5 mM $MgCl_2$, 3·75 mM $MnCl_2$, 2·5 mM dithiothreitol, 0·2 mM each of GTP, ATP and UTP and 0·2 mM [^{3}H]-CTP containing 0·5 μCi (New England Nuclear) and purified nuclei containing 7–10 μg of DNA in a final volume of 0·5 ml. The reaction mixture was incubated for 30 min at 37°C and reaction was terminated by the addition of 5 ml of cold 5% trichloroacetic acid (TCA). The precipitate was centrifuged and washed with 2·5% TCA containing 1 mM sodium pyrophosphate. Finally the precipitate was taken in Bray's scintillation fluid (10% naphthalene 0·4% PPO in dioxane) and counted in a Beckman liquid scintillation counter. Boiled nuclear preparation was used as blank. DNA was estimated by the method of Setaro and Morley (1977) using calf thymus DNA as standard.

Effect of trans-cinnamate on in vivo synthesis of PAL

The method described by Shirsat and Nair (1981) was used to determine the *in vivo* synthesis of PAL by isolating the [^{14}C]-PAL using affinity chromatography on phenylalanine conjugate of sepharose-4B prepared according to the method of Ussuf and Nair (1980). The PAL was eluted using 20 mM NH_4 acetate buffer, pH 9·3 and its activity as well as radioactivity were determined. The unabsorbed protein eluted at pH 6·0 was also collected and taken for determination of PAL activity and radioactivity.

Cell-free synthesis of PAL in wheat germ system

The wheat germ cell-free assay system for *in vitro* PAL synthesis was the same as described by Ussuf and Nair (1983). The PAL enzyme was separated from other translation products using affinity chromatographic purification on phenylalanine conjugated to Sepharose-4B.

Assay of PAL: The enzyme activity was determined according to the method of Zucker (1965). One unit of activity is the amount of enzyme which produced 0·01 increase in absorbance at 290 nm corresponding to 3·3 nmol of cinnamic acid (Zucker, 1968).

Values given in tables and figures are the mean of 5–6 independent experiments.

Results

In intact parenchyma of potatoes, RNA synthesis, represented by incorporation of [^{3}H]-uridine, took place at a slow rate till about 6 h after slicing. Further incubation of these tissues showed a linear increase in the incorporation upto 15–18 h followed by a steady state level. A typical incorporation pattern into RNA in excised potato parenchyma tissue is given in figure 1 (curve 1). The increase in PAL activity due to *de novo* synthesis of this protein was almost in parallel with the increase in RNA synthesis, although the maximum time taken for the enzyme synthesis was longer than for RNA synthesis (figure 1, curve 2). Treatment of this tissue with 3·5 mM trans-cinnamate reduced the RNA synthesis at every stage by about 50% (figure 1, curve 3). These observations suggest that the effect of trans-cinnamate may not be on DNA-dependent RNA polymerase but at the template level. Treatment of the tissue with 15 μg/g actinomycin D inhibited RNA synthesis by 65% (table 1).

Earlier studies reported by Shirsat and Nair (1981), showed that the inhibition of PAL synthesis observed in anaerobically incubated sliced potato parenchyma tissue was due to the accumulation of trans-cinnamate. Therefore, the effect of trans-cinnamate on RNA synthesis ensued during aging of excised parenchyma tissue was examined. The results given in table 1 showed that treatment of the tissue with trans-cinnamate (3·5 mM) within 2 h after slicing could inhibit RNA synthesis by about 50% level. But delaying the treatment upto 9 h *i.e.* when active RNA synthesis was linearly progressing the inhibition was only 20%. Treatment with trans-cinnamate at 12 h or beyond had no effect on RNA synthesis.

To examine whether trans-cinnamate acted at the template level, the transcriptional capacity of isolated nuclei from parenchyma was assessed. For this purpose nuclei were isolated from fresh 12 h aged and 12 h aged trans-cinnamate treated tissue. The DNA dependent RNA polymerase activity of these nuclei was tested without supplementing any template DNA for examining the availability of *in situ* DNA template for transcription. The results in table 2 showed that there was very little template availability in nuclear fraction isolated from fresh parenchyma tissue, while 5·5 fold increase in RNA synthesis was observed at 12 h. This increase was reduced to 3·4 (about 38% inhibition) fold in trans-cinnamate treated tissue nuclei suggesting that it may be interfering at the level of initiation of transcription.

In order to test whether the inhibition by trans-cinnamate is a general effect on RNA synthesis or it is specific for PAL-mRNA synthesis alone, its effect on *in vivo*
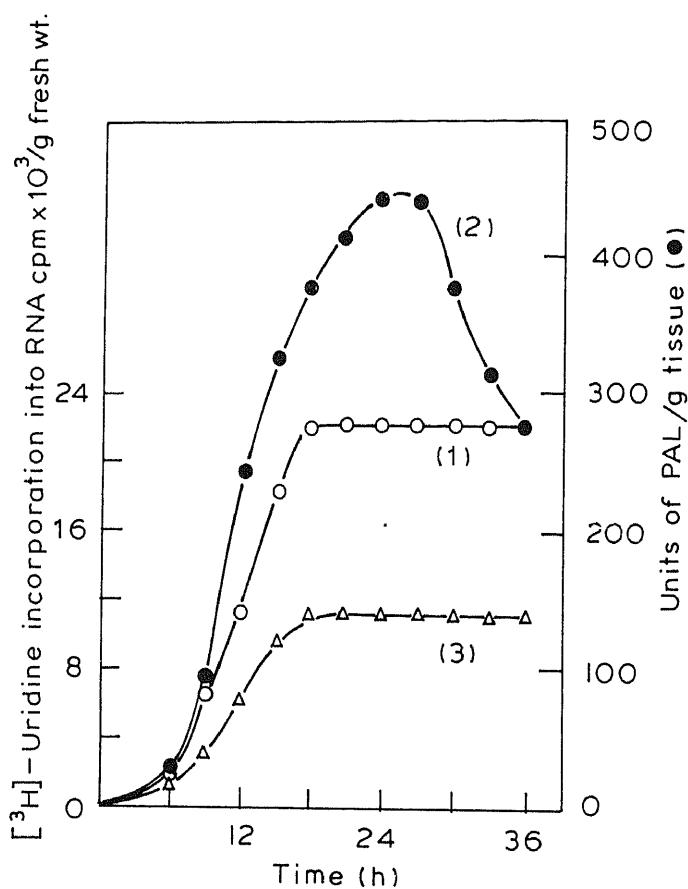
Figure 1. Curve 1 and curve 3 show incorporation of [^{3}H]-uridine into total RNA in aging control and trans-cinnamate treated (3·5 mM) potato parenchyma tissues, respectively. RNA was isolated by phenol extraction method at different time intervals. Curve 2. Time course of development of PAL in the aging potato parenchyma tissues. PAL was assayed according to the method described earlier (Shirsat and Nair, 1981).

incorporation of [^{14}C]-leucine into parenchyma tissue proteins was examined. Our earlier studies on the distribution of [^{14}C]-leucine into different tryptic peptides of PAL purified to homogeneity by affinity chromatography on phenylalanine conjugated sepharose-4B confirmed that the *in vivo* incorporation of [^{14}C]-leucine was a result of *de novo* synthesis of the enzyme (Shirsat and Nair, 1981). In this experiment parenchyma tissues were excised and treated with 3·5 mM trans-cinnamate at different time intervals as given in table 3. The tissues were incubated for a period of 24 h. Total proteins were isolated and aliquots were subjected to affinity chromatography. The fractions collected at pH 6·0 (washings) and pH 9·3 (eluate) were taken for radioactivity measurement and PAL activity determination. The result given in table 3 showed that trans-cinnamate treatment of the tissue did not have any effect on the incorporation of radioactivity into other proteins which were washed out at pH 6·0 but the synthesis of PAL, on the other hand, was

Table 1. Inhibition of RNA synthesis by actinomycin D and trans-cinnamate in aged potato parenchyma tissue.

Treatment	Amount of [^{3}H]-uridine incorporated into RNA cpm/g fresh wt.	Inhibition (%)
Control	23,400	
Actinomycin D (15 µg/g tissue)	8,150	65
Trans-cinnamate (3·5 mM)		
0 h	11,700	50
2 h	12,160	48
9 h	18,720	20
12 h	23,000	—

For treatment with trans-cinnamic acid, the tissue slices after incubation with [^{3}H]-uridine were suspended in 2 ml of 3·5 mM trans-cinnamate solution and kept for 30 min and then transferred to a moist filter paper spread on a petri dish and incubated for 16 h. The treatment was done the same way at different time intervals.
Experimental details are given under 'experimental' section.

Table 2. Effect of trans-cinnamate on nuclear DNA dependent RNA polymerase activity in aged potato parenchyma tissue.

Source of nuclei	pmol [^{3}H]-CMP incorporated into RNA/h/100 µg DNA
Fresh parenchyma	4
12 h aged parenchyma	22
12 h aged trans-cinnamate (3·5 mM) treated parenchyma	13·6

Nuclei were isolated from fresh 12 h aged parenchyma and 12 h aged trans-cinnamate treated parenchyma. The details for the isolation of nucleic acid and determination of DNA dependent RNA polymerase activity are given under 'experimental' section.

strongly inhibited at 0 h treatment with trans-cinnamate. Later additions resulted in reduced inhibition. Treatment of the tissue with trans-cinnamate at 18 h and subsequent incubation to 24 h had only negligible effect on the synthesis of PAL. These observations indicated the possibility that trans-cinnamate treatment may be impairing the transcription of mRNA required for PAL synthesis.

Our next attempt was to demonstrate that, the RNA isolated from aged trans-cinnamate treated parenchyma tissue after 16 h aging contained less mRNA compared to untreated control tissue, using a wheat-germ cell-free system described by Ussuf and Nair (1983). This system was capable of complete synthesis of enzymatically active PAL protein when total RNA isolated from aged parenchyma tissue was provided as mRNA judged by the affinity chromatographic separation of PAL from other translation products (Ussuf and Nair, 1980). The

Table 3. Effect of trans-cinnamate added at different time intervals on the synthesis of PAL and other proteins in potato parenchyma tissue.

Nature of treatment	pH 6·0 fraction (3·0 ml) Units of PAL	cpm	pH 9·3 fraction (3·0 ml) Units of PAL	cpm
None	0	93,250	1010	16,540
Trans-cinnamate treatment at				
0 h	0	92,120	0	800
3 h	0	91,980	48	1,430
6 h	0	95,020	290	8,010
9 h	0	94·610	410	10,260
12 h	0	93,730	524	11,680
15 h	0	96,010	730	13,410
18 h	0	94,560	980	14,980

10 g freshly excised parenchyma tissue blocks were soaked for 1 h in 2 ml solution of U-[^{14}C]-L-leucine 37·5 μCi (sp. act. 122 mCi/mmol). After 1 h the entire mass was transferred on to filter paper kept in a petri dish and incubated for 22 h in air and in light as control. The treatment with trans-cinnamate (3·5 mM) was done at different time intervals beginning from 0–18 h after treating the tissues with [^{14}C]-L-leucine. At the end of incubation period in each case the tissues were taken for isolation and purification of PAL by affinity chromatography on phenylalanine-conjugate of sepharose-4B as described by Shirsat and Nair (1981). The pH 6·0 and 9·3 fractions from affinity chromatographic column were assayed for PAL activity and radioactivity.

analysis of the cell-free translation products of total RNA isolated from freshly cut tissue, aged-tissue and trans-cinnamate treated aged tissue showed that translation ability of RNA isolated from trans-cinnamate treated tissue was reduced to about 66% after taking into account of the stable PAL-mRNA present in the tissue (table 4).

The homogeneity of PAL purified by affinity chromatography from *in vivo* and *in vitro* cell-free wheat germ system was determined on polyacrylamide gel

Table 4. Effect of trans-cinnamate on the synthesis of mRNA for PAL in aged potato parenchyma tissue.

Source of RNA used in cell-free systems	PAL units	[^{14}C]-Leucine incorporated into PAL
No RNA	0	70
Fresh parenchyma	7	7,175
16 h aged parenchyma	30	38,000
16 h aged trans-cinnamate treated parenchyma	14	17,020

The ability of RNA isolated from freshly excised parenchyma, 16 h aged parenchyma, trans-cinnamate treated 16 h aged parenchyma, was tested in wheat germ-cell free protein synthesizing system described by Ussuf and Nair (1983). PAL was separated from other translation products by affinity chromatography on phenylalanine conjugate of sepharose-4B.

electrophoresis (PAGE) under non-denaturing conditions. The PAL protein synthesized *in vivo* after incorporating [^{14}C]-leucine on purification by affinity chromatography was found to be homogeneous on PAGE. The single radioactive peak corresponded with the protein band (figure 2, curve 1). The translation product from wheat-germ system purified the same way showed PAL activity (table 4) and on PAGE exhibited a single radioactive peak coinciding with PAL protein (figure 2, curve 2). At the same time the purified translation product using mRNA from trans-cinnamate treated tissue showed only 33% incorporation of [^{14}C]-leucine (figure 2, curve 3). Addition of cycloheximide (20 μg/ml) into *in vitro* wheat–germ system inhibited PAL synthesis to about 80% (figure 2, curve 4).
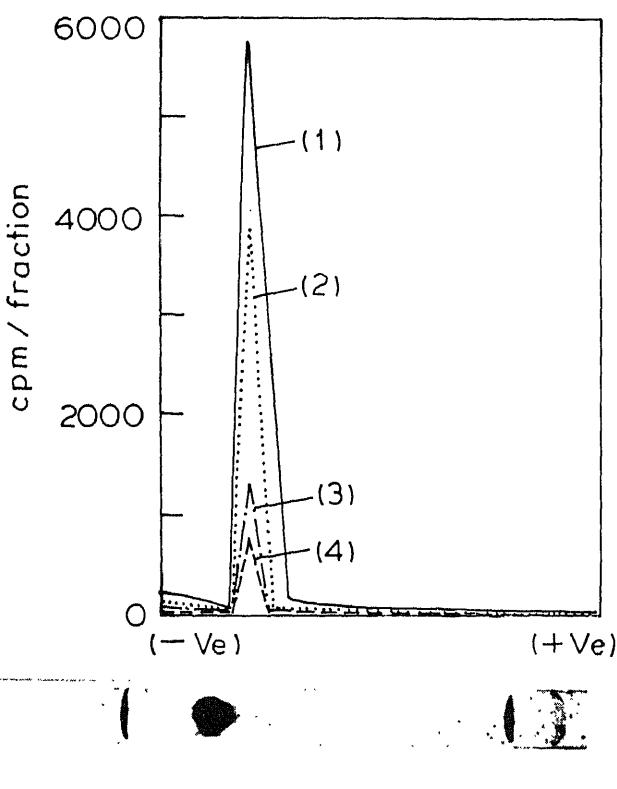
Figure 2. Identification of the homogeneity of PAL purified by affinity chromatography on phenylalanine conjugated sepharose-4B, on PAGE (gel photograph). Curve 1. *In vivo* incorporation of [^{14}C]-leucine into PAL during aging of sliced parenchyma tissue purified by affinity chromatography. Curve 2. *In vitro* incorporation of [^{14}C]-leucine using wheat germ system and RNA isolated from aged parenchyma into PAL purified by affinity chromatography. Curve 3. RNA used in this case was isolated from trans-cinnamate (3·5 mM) treated parenchyma tissue after aging. Curve 4. *In vitro* translation system was supplemented with cycloheximide 20 μg/ml.

PAGE was done on 7·5% acrylamide gels according to the procedure described by Davis (1964) at pH 8·3 using Tris-glycine buffer and staining was done with Coomassie brilliant blue.

Discussion

In the present study we have concentrated on the effect of the product of PAL, *viz.* trans-cinnamate, on the regulation of PAL synthesis. Earlier studies on the modulation of PAL by trans-cinnamate and other products were not very conclusive and these studies indicated that these compounds may not be directly acting on the transcriptional process but on post-transcriptional mechanism by affecting the processing or translation of PAL-mRNA (Lamb, 1979). The evidence presented here showed that like actinomycin D treatment, trans-cinnamate treatment inhibited the incorporation of uridine into RNA which exhibited time dependency. Maximum inhibition was observed only when the treatment was done within 2 h after slicing the tissue (table 1). If the modulation of PAL synthesis by trans-cinnamate is a post transcriptional effect it will be rapid and may not show any time dependency in regulating PAL enzyme synthesis (table 3). These observations in conjunction with the observation that there is a 38% reduction in the transcriptional ability of the isolated nuclei from trans-cinnamate treated tissue point out that trans-cinnamate may be interacting at template level limiting the transcription.

The tissues of plant storage organs like potato tubers are metabolically activated by slicing and exposure under aerobic condition to light (Kahl, 1974). Earlier studies from many laboratories have shown that the synthesis of rRNA, tRNA, and heterogeneous RNA are increased during the incubation period after slicing the tissue (Kahl, 1971; Oba *et al.*, 1978; Ishizuku *et al.*, 1981). The slicing also enhanced the chromatin bound DNA-dependent RNA polymerase activity and formation of polysomes (Kahl *et al.*, 1982; Oba *et al.*, 1982; Byrne and Setterfield, 1978). There were also changes in the synthetic rate, the content and size distribution of poly A^+ RNA and poly A^- RNA (Furuse *et al.*, 1983). These studies are suggestive that protein synthetic machinery is activated after wounding or slicing of potato tuber tissue.

It is well recognised that during excision and resultant wounding of potato tuber there is induction of PAL activity (Camm and Towers, 1977), leading to the formation of hydroxy cinnamate ester namely chlorogenic acid (Lamb and Rubery, 1976). There are also evidence to show that this induction of PAL is a result of *de novo* synthesis of this protein (Shirsat and Nair, 1981; Duchesne *et al.*, 1979; Loscheke *et al.*, 1981; Betz and Hahlbrock, 1979). Naturally for the production of active PAL enzyme transcription and translation of PAL gene is required, followed by post-transcriptional and post-translational modifications. From our current knowledge on the molecular structure of PAL enzyme, having a dehydroalanine residue at the active site (Hanson and Havir, 1970), post translational modification is a physiologically important event. In addition to this it is envisaged that limited proteolysis of PAL precursor may also take place to give the final holoenzyme. However, there is no conclusive demonstration of transcription of PAL gene in potato parenchyma tissue exhibiting *de novo* synthesis of PAL-mRNA.

While studying the *in vivo* synthesis of PAL enzyme (table 3) it was observed that the synthesis of PAL protein was almost completely inhibited in trans-cinnamate treated tissue, when the tissue was treated at 0 h, without affecting the synthesis of other proteins, although the inhibition of total RNA synthesis by trans-cinnamate was only 50%. The lack of inhibition of other proteins in trans-cinnamate treated

tissue may be partially due to the presence of stable mRNA in parenchyma tissue. Our earlier studies showed that this tissue responds maximally to cutting induced PAL synthesis (Pendharkar and Nair, 1977). So one cannot eliminate also the possibility that trans-cinnamate may be altering only the transcription of PAL-mRNA. The presence of stable mRNA for PAL in freshly cut parenchyma tissue is evident from the observation that total RNA isolated from this tissue is capable of translation of PAL enzyme in wheat germ system. The increase in the translational ability of 12 h aged parenchyma tissue is attributed to the enhanced synthesis of PAL-mRNA which is inhibited by trans-cinnamate treatment. However, further work is needed to prove that trans-cinnamate blocks the *de novo* synthesis of PAL-mRNA.

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Solubilization and interaction of α-tocopherol in water-aerosol OT-isooctane systems

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Abstract. Solubilization and interaction of α-tocopherol into bis(2-ethylhexyl)sulphosuccinate sodium salt microemulsion systems have been studied by temperature dependent phase transition, viscosity and nuclear magnetic resonance studies. Tocopherol being an amphiphilic molecule dissolves into the interfacial surfactant monolayer of the microemulsion droplets. The dissolution leads to an enhancement of the rigidity of the surfactant monolayer as studied by the increase in mixing and phase transition temperatures of the microemulsion droplets. Solubilization of tocopherol into microemulsion droplets causes an increase in the effective size of the droplet and as a consequence, the inter-droplet interactions are also increased. The water binding capacity of the surfactant (bis(2-ethylhexyl)sulphosuccinate sodium salt) is reduced due to solubilization of tocopherol as is evidenced from the downfield shifts of water proton magnetic resonances. In the presence of the dissolved electrolytes into the aqueous core, tocopherol is squeezed out of the microemulsion droplets increasing the membrane fluidity and permeability.

Keywords. Solubilization; aerosol OT; α-tocopherol.

Introduction

Vitamin E, mainly in the form of α-tocopherol, is nature's best fat-soluble antioxidant (Scott, 1978). Whether its function in plants and animals lies solely in its antioxidant properties or is mediated through some other mechanisms still remains to be elucidated. The hydrocarbon chain of α-tocopherol is found to have physico-chemical interactions with the fatty acyl chains of polyunsaturated phospholipids which cause stabilization of the membrane (Lucy, 1972). The interaction of α-tocopherol with biological membranes has been studied in various model membrane systems using nuclear magnetic resonance (NMR), electron paramagnetic resonance and fluorescence techniques (Schmidt *et al.*, 1976; Cushley and Forrest 1977; Fukuzawa *et al.*, 1981). The results suggest that the membrane fluidity and permeability are significantly affected by α-tocopherol, although no detailed information on its interaction with membranes has been obtained. Recent studies on α-tocopherol incorporated in normal micelles (Yamaguchi *et al.*, 1980) have indicated that the 'soft' hydrocarbon core of the micelle becomes 'hard' due to

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Abbreviations used: NMR, Nuclear magnetic resonance; AOT, bis(2-ethylhexyl)sulphosuccinate sodium salt.

introduction of α-tocopherol. The packing of amphiphiles in bilayer biological membrane is significantly different from that of micelles and hence the results obtained from the interaction with normal micelles cannot be quantitatively applied to the biological membranes.

The surfactant monolayer of reverse micellar droplets has been more suitably used (Maitra and Patanjali, 1986) as a model membrane system for solubilization studies. The advantage of such a system is that the amphiphile aggregates are simple and isotropic and the surfactant molecules in a large *w/o* microemulsion droplet have a packing factor comparable to that of lipids in biological membranes (Mitchell and Ninham, 1981). Large amphiphilic organic molecule such as α-tocopherol will have a distribution between the bulk solvent and the hydrophobic shell of the surfactant monolayer of the microemulsion droplet. Any physico-chemical change relating to the droplet containing solubilized tocopherol may be thought of as due to interaction between the surfactant molecule and tocopherol and the results would enable one to gain an insight into the tocopherol solubilization into biological membrane.

Experimental

Materials

Bis(2-ethylhexyl)sulphosuccinate sodium salt (AOT) was procured from Fluka AG and was carefully purified according to the procedure described elsewhere (Maitra and Eicke, 1981). dl-α-Tocopherol was also purchased from Fluka AG and was used without further purification. Dry isooctane and double distilled water were used.

Ternary solution

H_2O/AOT/i-C_8H_{18} solutions were prepared by dissolving appropriate weights of AOT in isooctane (0·1 M) and adding to this solution a precise volume of water. After proper sonication a stable microemulsion was formed in which the molar ratio of water to AOT was expressed by w_o. To study the effect of electrolytes on the solubilization system, sodium chloride of known concentration was added, and the w_o was then calculated accordingly.

Addition of the fourth components

Known concentration of α-tocopherol in isooctane was added to H_2O/AOT/ isooctane system to maintain the desired molar ratio (R) of α-tocopherol to AOT in the system.

Viscosity measurements

The measurements were performed with an Ubbelohde viscometer under high precision thermostatic control ($\pm 0\cdot1^\circ$C).

NMR spectroscopy measurement

NMR spectra were taken in a Perkin Elmer R-32, 90 MHz proton NMR Spectrometer. All chemical shifts were measured with reference to TMS resonance and are reported in ppm.

Results and discussion

Solubilization diagrams

One of the important studies in water-in-oil microemulsion system is the water solubilization diagram, in which a plot of the water content (indicated by w_o) against temperature shows a triangular shaped region represented by optically isotropic, thermodynamically stable, clear, low viscous microemulsion phase. The positions of the upper and lower boundary curves of the microemulsion region are determined by the composition of the microemulsion, particularly by the nature of the interfacial surfactant monolayer of the microemulsion droplets (Eicke, 1979). The surfactant monolayer of a microemulsion droplet mimics a biological membrane and any interaction of an extramembraneous component with the biological membrane can be suitably studied from the solubilization of the former into the microemulsion system (Patanjali, 1985). The change in the interfacial rigidity of the surfactant monolayer due to the addition of a fourth component would be reflected in the trends of lower and upper boundary curves of the solubilization diagram which correspond to the mixing and phase transition temperatures respectively at a particular droplet size (Eicke, 1979). Figure 1 shows the influence of tocopherol on the water solubilization diagrams of water-AOT-isooctane microemulsion system. At a particular w_o, the microemulsion containing
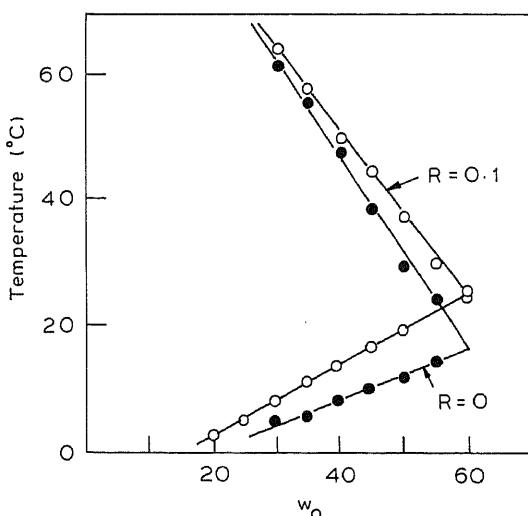
Figure 1. Solubilization diagram of the water-AOT-isooctane systems with (R = 0·1) and without (R = 0) tocopherol (where R is molar ratio of tocopherol to AOT).

tocopherol needs a higher temperature to dissolve water than that without the additive. This increase in mixing temperature for the system containing tocopherol shifts the lower boundary curve towards higher temperature region. Tocopherol, an amphiphilic molecule, is expected to be dissolved into the surfactant monolayer of the droplet although it would maintain a distribution between the droplets and the bulk solvent. Without going into the quantitative details of such distribution it can be said that a higher mixing temperature due to the presence of tocopherol indicates that more energy is required for an isothermal transfer of water from the two-phase region to the microemulsion region. Thus, tocopherol affects the microemulsion in such a way as to increase the interfacial energy under isothermal conditions. The shifting of the upper boundary curve also towards the higher temperature region upon the addition of tocopherol shows that the phase transition from the so called 'closed' aggregates to 'open' aggregates of the microemulsion droplets (Eicke and Markovic, 1981; Eicke, 1979), needs higher energy. A combined analysis of both the results clearly indicate that tocopherol directly influences on the rigidity of the surfactant monolayer of the droplets. The result is in conformity with earlier investigations which showed that tocopherol significantly decreases the fluidity and permeability of biological membranes (Rice-Evans and Hochstein, 1981). When an additive modifies the environment of the surfactant monolayer by dissolving into and interacting with it, then, any change in the interfacial activity will be indicated by the change in the kinetics of the phase transition process. If membrane rigidity is responsible for the phase transition process, then an Arrhenius plot of the logarithm of hydration (log w_o) against the inverse of the corresponding phase transition temperature as shown in figure 2 would provide activation energy values for the dynamic phase transition processes. These have been calculated to be 18·02 kcal m^{-1} and 14·45 kcal m^{-1} for the microemulsion system with and without tocopherol respectively (table 1). The differences in the activation energies would indicate an additional rigidity of AOT surfactant monolayer of microemulsion droplets when tocopherol is dissolved into it.
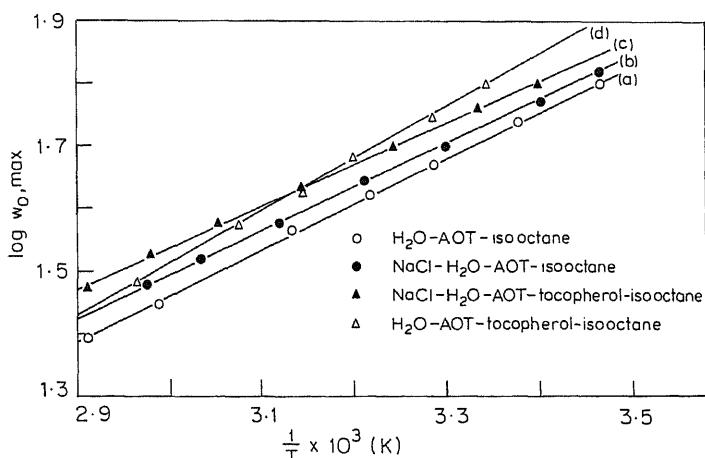
Figure 2. Arrhenius plot of Log w_o vs. inverse of corresponding phase transition temperature (1/T).

Table 1. The values of shape factor, λ_p, in AOT/H$_2$O/i-C$_8$H$_{18}$ solutions at $w_o = 20$ with and without α-tocopherol determined from intrinsic viscosity $[\eta]$ and apparent specific volume.

System	$-\Delta E$ (KCal/mol)	$\bar{V}_2$ (cm^3/g)	$[\eta]$ (cm^3/g)	λ_p	Prolate $P>1$	Oblate $P<1$
H$_2$O/AOT/i-C$_8$H$_{18}$	14·45	1·008	2·08	2·77	1·88	1·88
NaCl/AOT/i-C$_8$H$_{18}$	14·96	—	—	—	—	—
H$_2$O/AOT-Tocopherol/i-C$_8$H$_{18}$	18·02	0·954	2·88	4·02	2·16	0·49
H$_2$O/AOT-Tocopherol/i-C$_8$H$_{18}$	—	0·935	2·96	3·16	2·33	0·43
NaCl/AOT-Tocopherol/i-C$_8$H$_{18}$	13·54	—	—	—	—	—

Effect of electrolytes

When an electrolyte such as sodium chloride is dissolved into the aqueous core of microemulsion droplets containing tocopherol, the water solubilization behaviour of the 5 component system *viz*. NaCl-H$_2$O-AOT-Tocopherol-isooctane is changed, as is shown in figure 3. The phase transition temperature indicated by the upper boundary curve is found to be shifted to lower values and the activation energy for the phase transition process is considerably reduced (table 1). These observations can be interpreted in the following terms. It is known that an increase in sodium ion concentration in the aqueous core of the microemulsion droplets leads to a decrease in the electrolytic dissociation of the AOT sodium sulphosuccinate at the interface (Eicke, 1980). As a result, the electrostatic repulsive force between the AOT head groups is considerably reduced such that the AOT molecules at the interface could come closer together with a consequent reduction in the effective head group area in the aggregated system. Under such circumstances, a bulky molecule like tocopherol would be sterically inhibited and would tend to be squeezed out of the surfactant monolayer of the droplet. The significant lowering of mixing temperature due to the addition of sodium chloride in the H$_2$O-AOT-tocopherol-isooctane system as shown in the figure 3 is a clear indication that the
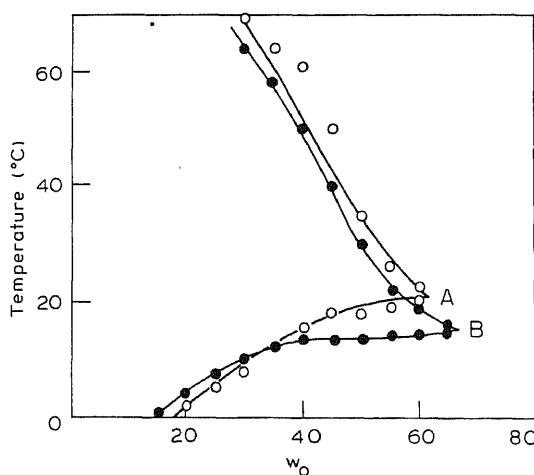
Figure 3. Solubilization diagram of H$_2$O-AOT-tocopherol-isooctane with (A) and without (B) electrolyte (NaCl).

membrane rigidity attained by the droplets due to solubilization of tocopherol is considerably reduced, once again, in the presence of sodium chloride. The decreased rigidity of the surfactant monolayer of the droplets is also evidenced from the decrease in activation energy of the phase transition process when sodium chloride is added to the microemulsion system containing tocopherol.

Viscosity studies

Tocopherol being a bulky molecule may deform the shape and change the morphology, of the aggregated system when it is dissolved into it. Viscosity studies of H_2O-AOT-isooctane containing tocopherol would provide valuable informations about the deformations of the parent spherical droplets as well as interdroplet interactions. Moreover, the hydrophobic part of the tocopherol molecule is very large and the effective length is longer than that of the AOT molecule. Therefore, dissolution of tocopherol into the droplet with the polar hydroxyl group being directed towards the inner aqueous core of the droplet may induce an imbalancing volume effect of the latter affecting the viscosity parameters (Bansal *et al.*, 1980). Since the relative viscosity (η_{rel}) is dependent on AOT concentration, volume fraction of the droplets and temperature, the experiment was conducted at constant temperature (308 K) and at constant AOT concentration in order to observe the dependence of η_{rel} on droplet volume fractions (ϕ). ϕ, however, is a direct measure of the amount of the added water in the system *i.e.* w_o. A plot of η_{rel} *vs* w_o is shown in figure 4. The curves show that the relative viscosities increase slowly with the increase in w_o while at higher w_o the increment is abrupt. The relationship between the relative viscosity and the concentration of hydrated surfactant (with and without tocopherol as the case may be) was studied under the assumption that the dilution of the system affects primarily the interparticle distance and their interaction. Figure 5 illustrates the relationship between η_{rel} and concentration of hydrated micelles at $w_o=20$ and at 308 K. The higher η_{rel} values for the system containing tocopherol indicates enhanced interparticle interaction. This is not
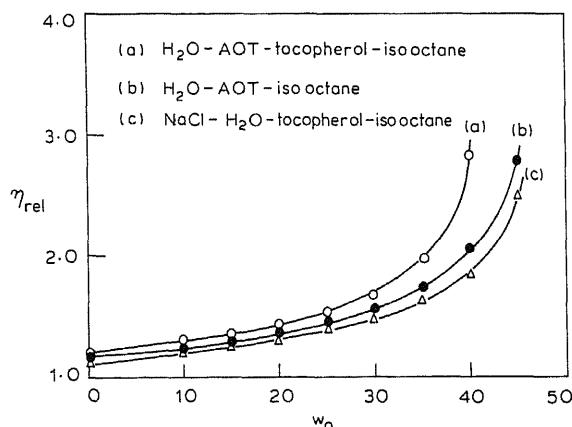
Figure 4. Relation between relative viscosity η_{rel} and w_o at constant concentration of AOT at 35°C.
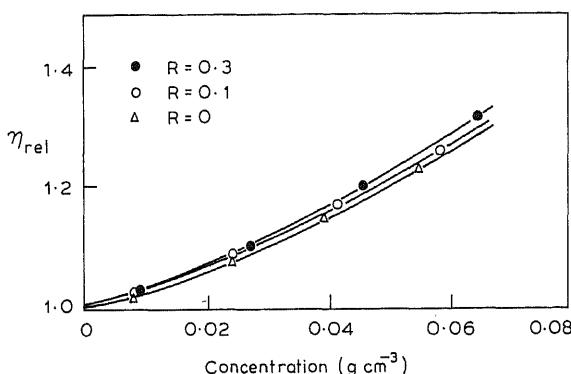
Figure 5. Dependence of relative viscosity, η_{rel}, on hydrated droplet concentration in H_2O-AOT-tocopherol-isooctane at 35°C and at constant w_o (*i.e.* = 20).

surprising when one considers the increase in effective volume of the droplets due to solubilization of tocopherol whose hydrocarbon chain length is longer than that of AOT molecule. Another interesting observation is that the relative viscosity of the water-AOT-tocopherol-isooctane system is reduced considerably when sodium chloride is introduced into the aqueous core of the droplet. This is definitely an indication of the lowering of the interparticle interaction among the droplets which is caused, as explained earlier, due to expulsion of tocopherol molecules from the surfactant monolayer of the droplet. The observation further supports the view that extra electrolyte into the aqueous core of the droplet has a contraction effect on the surfactant monolayer by which the dissolved tocopherol molecules are squeezed out of the droplets with a consequent reduction in the effective volume of the droplets.

The equation relating the intrinsic viscosity $[\eta]$ and the volume fraction of the dispersed particles, ϕ, can be described in the dilution limit by (Einstein, 1906).

$$\eta_{rel} = \eta_o \ (1+2\cdot5\phi), \tag{1}$$

which can also be written in the form

$$[\eta] = 2\cdot5/\rho, \tag{2}$$

in which the intrinsic viscosity $[\eta]$ is defined as equal to η_{sp}/C where $C \rightarrow 0$ and ρ is the density of the colloidal particles. For a perfectly spherical droplet $[\eta]_{c=0}$ is approximately equal to $2\cdot5$. For an asymmetric non-solvated particle which can be represented as an ellipsoid of revolution of volume V and axial ratio p, the intrinsic viscosity and the apparent specific volume V are related by equation (Markovic, 1980).

$$[\eta] = V\cdot\lambda_p, \tag{3}$$

in which λ_p is a function of axial ratio p. The apparent specific volume V of the solute can be replaced by the partial specific volume $\bar{V}_2$ of the solute particles. In figure 6, η_{sp}/C is plotted against the particle concentration. The intrinsic viscosity was calculated by intrapolating the curve to $C = 0$ as shown in figure 6. The viscosity parameters for the AOT microemulsion systems with and without
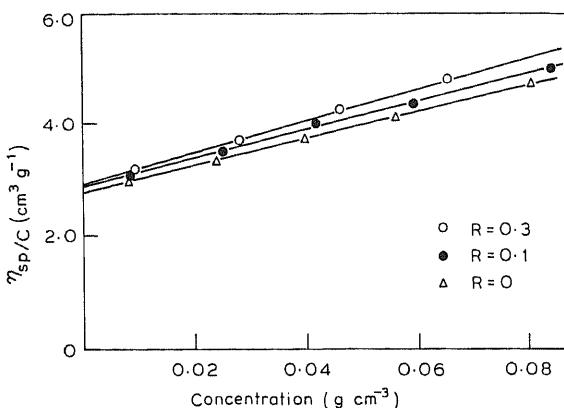
Figure 6. Dependence of reduced viscosity, $\eta_{sp/c}$ on the concentration of hydrated droplet of H_2O-AOT-tocopherol-isooctane systems at 35°C and constant w_o (*i.e.* = 20).

tocopherol and sodium chloride are shown in table 1. Since there is no easy experimental method available for directly estimating the shape of the particle in microemulsions, the assumption has been made that the particles are ellipsoids of revolution or at least of equivalent shape. The shape of the microemulsion droplet in the reference system at $w_o = 20$ is more or less spherical, as observed from $[\eta]_{c=0}$ measurements (Maitra *et al.*, 1983). Any subsequent deformation of these spheres by the dissolution of tocopherol would then tend to produce ellipsoid of revolution. The particle shape factor calculated for various systems are shown in table 1. For each value of λ_p the corresponding two axial ratios—one prolate with $p > 1$ and the other with $p < 1$ are taken from the literature (Sadron, 1942), and are shown in the same table. These values would be a reflection of the deformation of an otherwise nearly spherical droplet upon the solubilization of tocopherol.

NMR studies

Water molecules within the aqueous core of the reverse micelles and of *w/o* microemulsion droplets are highly structured (Wong *et al.*, 1977). Molecules at the interface near the polar head groups are immobilised by hydrogen bonding and form the distinctly bound water layer. Water molecules at the bulk of the aqueous core are relatively mobile and exhibit properties similar to that of normal water. In NMR water proton chemical shift experiment in such a microemulsion system, only one resonance peak for water protons is observed, indicating that there exist a fast exchange of protons between the bound water region and the relatively free water region in the aqueous core. An increase in the water content from $w_o = 4$ to $w_o = 50$ gives only one resonance peak and the figure 7 shows the change of the water proton resonance peaks at various w_o. This change is more dominant in the microemulsion region rather than in the micellar region for various AOT systems. Upon the solubilization of tocopherol, the water proton resonances exhibit a downfield shift compared to those in the reference system. Upfield shifts of these proton resonances are observed once again when sodium chloride is added to the aqueous core of the microemulsion droplets. It was earlier established that an
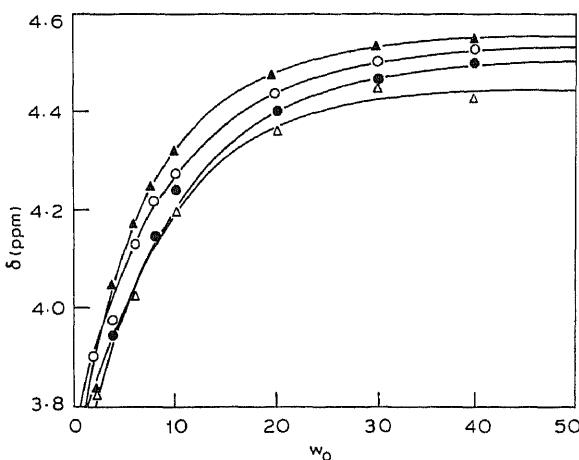
Figure 7. Variation of water proton chemical shifts against w_o.
(▲), H₂O-AOT-tocopherol-isooctane; (○), NaCl-H₂O-AOT-tocopherol-isooctane;
(●), NaCl-H₂O-AOT-tocopherol; (△), H₂O-AOT-isooctane.

increase in bound water fraction in the aqueous core leads to the water proton chemical shifts more towards higher fields (Maitra, 1984). Downfield shifts of water proton resonances due to the addition of tocopherol may, therefore, indicate that the fraction of bound water in the aqueous core is decreased. It is thus concluded that the tocopherol at the interface decreases the hydrophilicity of the surfactant polar groups. The plausible explanation for this may be that the water binding capacity of the polar groups of AOT through hydrogen bonding is considerably reduced due to intermolecular hydrogen bonding between AOT polar groups and hydroxyl group of tocopherol. The surfactant molecules regain their hydrophilicity when sodium chloride is present in the aqueous core of the droplets as is evidenced from the upfield shifts of water proton resonances once again (figure 7).

Conclusion

From the solubilization characteristics of tocopherol into the water-AOT-isooctane system, it may therefore be concluded that the compound is partitioned into the surfactant monolayer of the droplet and causes change in the properties of the aggregated system. The surfactant monolayer becomes harder due to the presence of tocopherol and the water permeability of the monolayer is considerably decreased as is evidenced from the rise in mixing temperature in the solubilization diagram. An increase in the activation energy for the phase transition process is indicative of the enhanced rigidity of the surfactant monolayer. Tocopherol being a bulky molecule, causes a change in the packing of the AOT molecule in the aggregated system and its solubilization into the microemulsions leads to a change in the shape and size of the droplets. In the presence of sodium chloride, the dissolved tocopherol molecules are squeezed out of the microemulsion droplets and the surfactant monolayer of the droplet becomes softer and prone to water permeation. Tocopherol, therefore, does not behave like a cosurfactant in the

classical sense. Rather, it interacts with the amphiphilic molecules in such a way that the water binding capacity of the surfactant polar groups is decreased with a concomitant increase in membrane rigidity.

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A method for the isolation of intact Sertoli cell–germ cell associations from rat seminiferous tubules and their further partition into Sertoli cell and germ cell fractions

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Abstract. A technique is described for obtaining a Sertoli cell-enriched and a germ cell-enriched fraction from immature rat testes. Sertoli cell–germ cell associations were obtained by incubating washed seminiferous tubule fragments with Collagenase and Pancreatin. They were then manually dissociated into a suspension comprising Sertoli cells as well as the various germ cell types characteristic for a given day of ontogeny. Fractionation into a Sertoli cell-enriched fraction and a germ cell-enriched fraction was effected by centrifugation following layering over a stepwise gradient of Ficoll-400. While the time-span compares favourably with other procedures reported in the literature, it is believed this is the first time a method is described that enables the simultaneous recovery of both the Sertoli cells and the germ cells.

Keywords. Rat seminiferous tubules; Sertoli cells; germ cells; isolation.

Introduction

It is now generally accepted that the Sertoli cells have an important role to play in germ cell development and differentiation (Fritz, 1978; Russell, 1980). Furthermore, the view that the orderly progression of spermatogenesis is dependent upon a continuous interplay between the 'Sertoli cell compartment' and the constituents of the 'germ cell compartment' has attracted much attention (Ritzén, 1983). Paradoxically enough, the presumed nexus between these two compartments appears to have had little impact, until recently (Ritzén *et al.*, 1982), on the design of investigations aimed at unraveling the molecular basis of spermatogenesis and its regulation (Meenakumari, 1984). For the most part, *in vitro* biochemical studies have utilized either cultured Sertoli cells or isolated germ cell types.

We were interested in an investigation of possible Sertoli cell–germ cell interactions, specifically in relation to protein synthesis, during the ontogeny of spermatogenesis in the colony-bred rat. Consequently, our experimental design (Meenakumari, 1984) called for incubation *in vitro* of either seminiferous tubules *per se* or of intact 'Sertoli cell–germ cell associations' (SGAs) isolated therefrom, to be followed, at the appropriate time, by separation into a Sertoli cell-enriched and a germ cell-enriched fraction for purposes of analyses or further fractionation. In the absence of a suitable published procedure meeting such a requirement, it became necessary to develop one.

Abbreviations used: SGAs, Sertoli cell–germ cell associations; SCS, single cell suspension.

Materials and methods

Animals

The male rats used in this study were from our Holtzman-derived colony maintained under standard housing conditions ($24 \pm 1°C$; 14 h light-10 h dark schedule; free access to water and commercial pelleted rat diet). For reasons dealt with elsewhere (Meenakumari, 1984), attention was focussed on immature animals of postnatal age, days 8, 14, 18, 23 and 30, respectively. Only rats born on the same day were used for a given experiment. Testes (6–30) were employed depending on the selected age group.

Chemicals and reagents

Collagenase (Type 1) and Pancreatin (porcine, Grade VI) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Ficoll-400 was bought from Pharmacia, Uppsala, Sweden. All other reagents were purchased locally and were of analytical grade purity.

Buffers

Buffer A contained: sodium chloride-97·6 mM; potassium chloride-25 mM; glucose-8·3 mM; L-glutamine-5 mM; Tris (Tris hydroxymethyl amino methane)-10 mM; penicillin-50 units/ml; streptomycin-50 μg/ml and phenol red-0·008 mM. The pH was adjusted to 7·3 at 10°C using 2 N HCl.

Buffers B, C and D had the same composition as Buffer A but were adjusted to pH 7·3 at different temperatures (B-28°C; C-32°C; D-25°C). In addition, B was made 26 mM with respect to $CaCl_2$ and D, 1 mM with respect to EDTA. Buffer C was used whenever tissue–seminiferous tubule segments/SGAs–had to be incubated.

Isolation of SGAs

The following procedure modified from Welsh and Wiebe (1975) was used. Animals were anesthetized and the testes removed and kept in Buffer A till end of collection. Decapsulated testes were suspended in Buffer B and passed gently through a 1 ml hypodermic syringe 10–12· times. With the rapid settling of the seminiferous tubules, the supernatant was decanted. This step was repeated once more. The washed tubules were cut into 0·5–1·0 mm long fragments, suspended in Buffer B containing Collagenase and Pancreatin (1 g tissue/10 mg of each enzyme/20 ml Buffer B) and incubated at 28°C in a Dubnoff metabolic shaker water-bath at 140 oscillations per min for an appropriate period of time. The Leydig cell-peritubular cell aggregates formed in this step were removed by aspiration to yield a preparation consisting of SGAs. These were allowed to settle and the supernatant containing the enzymes, single cells and small-sized SGAs was removed by aspiration. The SGAs were washed several times with Buffer D and suspended in a known volume of the same buffer.

Preparation of single cell suspension

The washed suspensions of SGA, derived from testes of defined age, were processed as follows to obtain, in each case, a single cell suspension (SCS): (i) 8 and 14–passed 30 times through a Pasteur pipette; (ii) 18–passed 30 times through Pasteur pipette and twice through a hypodermic syringe fitted with a 22-gauge needle, at the rate of 1 ml per second; (iii) 23 and 30–passed 30 times through Pasteur pipette and 8–10 times through the hypodermic syringe. Frothing was avoided at all times.

The cells in SCS were counted using a haemocytometer.

Separation of cell types

The SCS containing both Sertoli cells and the various germ cell types was mixed with an equal volume of a 6% Ficoll-400 solution made up in Buffer D. Aliquots containing 3 to 4×10^6 cells were layered over 30 ml of a stepwise Ficoll gradient (5%–10%–15% or 5%–10%–15%–20%) and centrifuged in a Spinco SW-25 rotor for 10 min at 25°C. The cells formed discrete bands based on their densities. Each of the resultant fractions was removed by aspiration, mixed with an equal volume of Buffer D and centrifuged to recover the cells. Each fraction was washed twice to remove the Ficoll and finally suspended in 0·5 ml of Buffer D.

Identification of cell types

A small drop from each fraction was spread over a clean glass slide and fixed immediately in formaldehyde vapour, at room temperature. The cells were stained by the Feulgen technique. The various cell types in each fraction were identified according to Meistrich *et al.* (1973) and counted.

Cell viability

The viability of cells was monitored at every step by the Trypan blue exclusion test.

Purity of SGAs

Leydig cell contamination was checked by the histochemical method described by Welsh and Wiebe (1975). The peritubular cells were identified by periodic acid-Schiff staining as well as by their characteristic spindle shaped appearance (Tung and Fritz, 1977).

Results

Isolation of SGAs

The steps described above enabled recovery of intact, viable SGAs free of peritubular and Leydig cells. The sequential removal of these two cell types is shown in figures 1–4. Passage through the hypodermic syringe freed the seminiferous tubule fragments of perivascular–Leydig cells (figure 1). The peritubular cells as well as the peritubular–Leydig cells were removed during the treatment with the enzymes (figures 2 and 3). The duration of exposure to enzymes
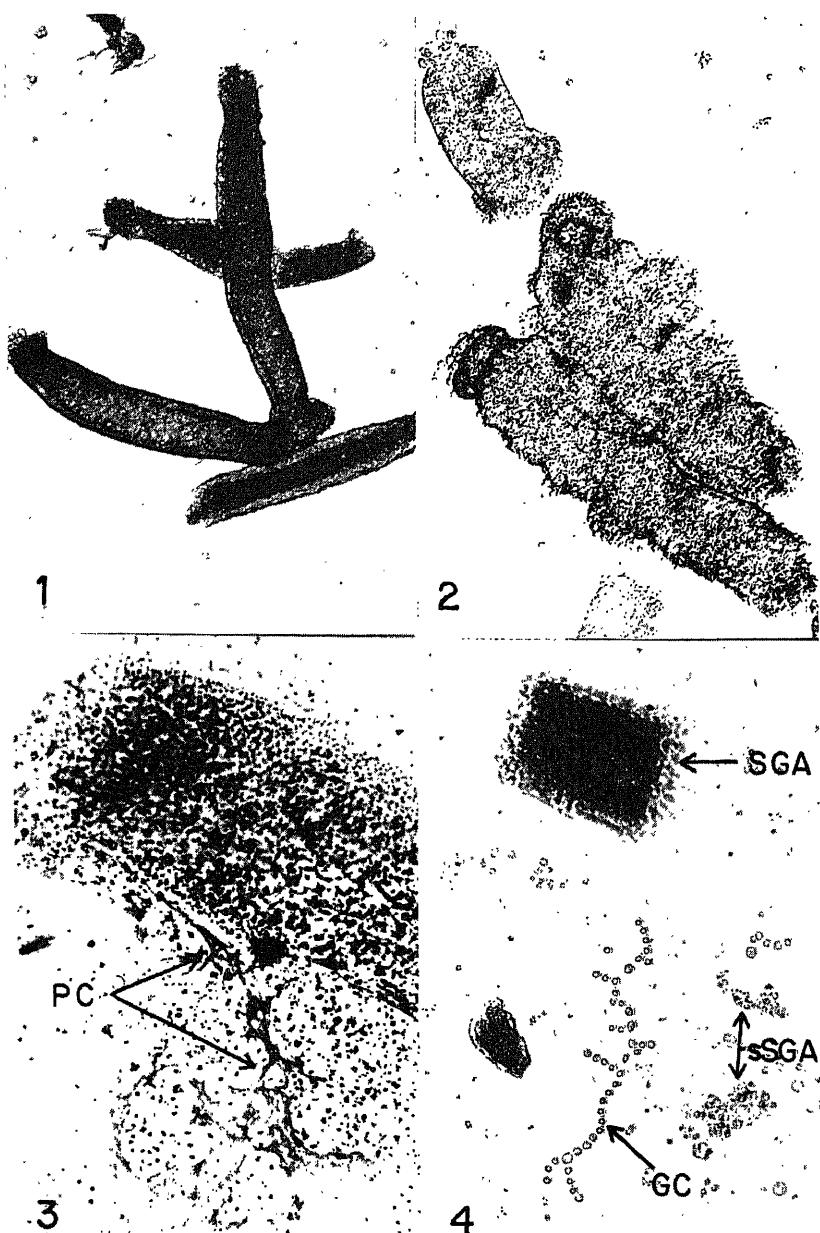
Figures 1–4. Sequence in the conversion of seminiferous tubule fragments into a pure preparation of SGAs, as monitored by microscopy. (1–3), Transmission micrographs; (4 phase contrast micrograph. **1.** Tubular fragments freed of perivascular-Leydig cells by initial washing and mincing (×125). **2.** Appearance of tubule fragments half-way through incubation with Collagenase and Pancreatin (×125). **3.** Tubular fragment during the las phase of enzyme digestion. Material was fixed in formaldehyde vapour and stained by the Feulgen method. Aggregated peritubular cells are seen in the areas marked PC (×77) **4.** End of enzyme digestion: The preparation is free of Leydig cells as well as peritubula cells. SGA: a typical Sertoli cell–germ cell association. Note the presence of small SGA clusters (sSGA) and strings of germ cells (GC). Isolated single cells are also seen (×123)

was dictated by the age of the donor animals, being 15–20 min for day 8, 25 min for day 14, 35 min for day 18, 45 min for day 23 and 60 min for day 30.

SCS

Conversion of the SGAs from 8- and 14-day old testes to single cell suspensions was readily effected, in as much as, at this early stage of ontogeny, the association of the limited number of germ cells with the Sertoli cells was tenuous. This was in contrast to the later stages—especially following their migration to the adluminal compartment.

Cell separation

The pattern of cell separations obtained on centrifugation of the SCSs on the Ficoll gradients is shown in figures 5–9 (insets). The distribution of the various cell types in the different fractions is shown for each age group (figures 5–9). The purity of the Sertoli cell and the germ cell fractions.for the specified age groups as well as the extent of cross-contamination are presented in table 1.

Table 1. Purity of fractions.

Age in days	Sertoli cell fraction (%)	Germ cell in Sertoli cell fraction (%)	Germ cell fraction (%)	Sertoli cell in germ cell fraction (%)	Peritubular cells (%)	Leydig cells
8	96	4	91	1	Nil	—
14	92	8	77·27	21·53	1·19	—
18	85	15	84·84	14·39	0·9	—
23	42	58	76·77	22·42	0·8	—
30	78	22	88·39	10·84	0·76	—

Discussion

In the past few years, several methods have been described for the isolation and culture of Sertoli cells from rats (Dorrington *et al.*, 1975; Steinberger *et al.*, 1975 a,b; Welsh and Wiebe, 1975; Galdieri *et al.*, 1981). As for germ cells, methods have been described for obtaining preparations enriched with respect to one or more cell types from immature as well as sexually mature rats (Go *et al.*, 1971; Davis and Schuetz, 1975; Grimes *et al.*, 1975; Platz *et al.*, 1975; Alemán *et al.*, 1978; Beckman *et al.*, 1978; Nakamura *et al.*, 1978). Since our investigations (Meenakumari, 1984) required recovering both the Sertoli cell fraction and the germ cell fraction from a given source, preferably in quantitative yields, it became necessary to devise a fresh procedure.

The present method was modified from the technique originally described by Welsh and Wiebe (1975) for obtaining Sertoli cells from immature rat testes. Not surprisingly, they discard a germ cell-rich fraction (fraction 2) during the initial washing. Since such heavy loss of germ cells had to be obviated for our purposes, the seminiferous tubule suspension, obtained by stripping off the tunica albuginea,
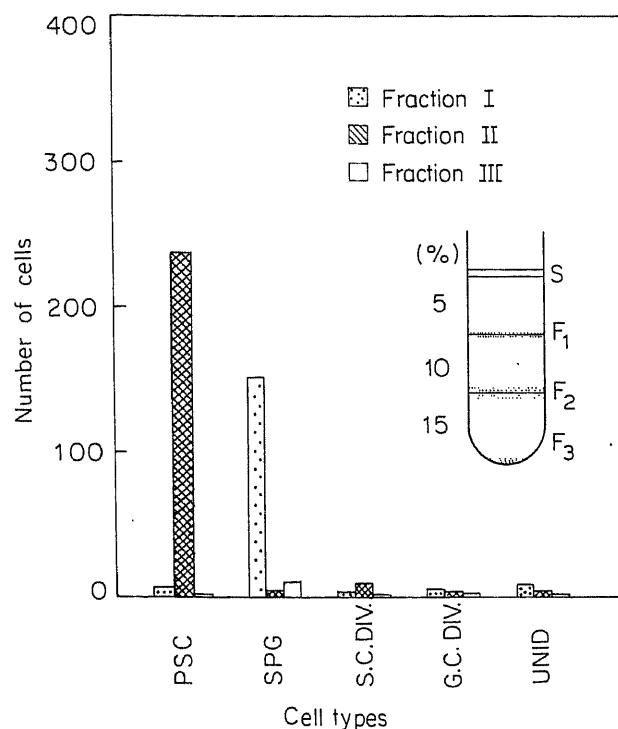
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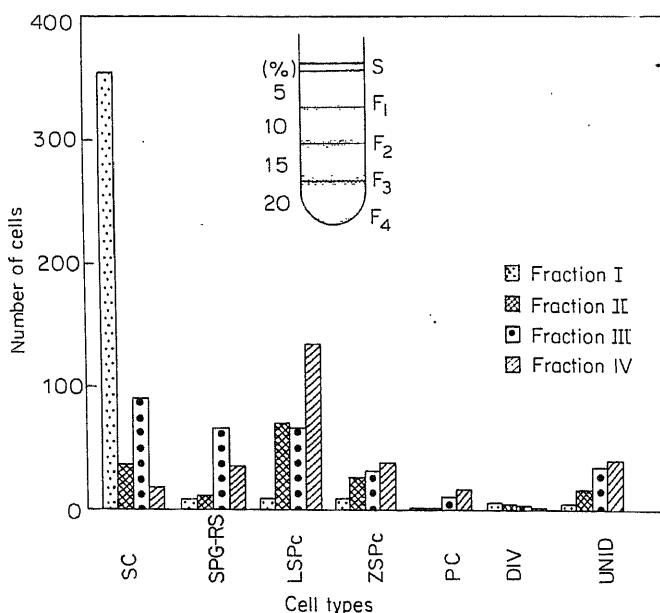
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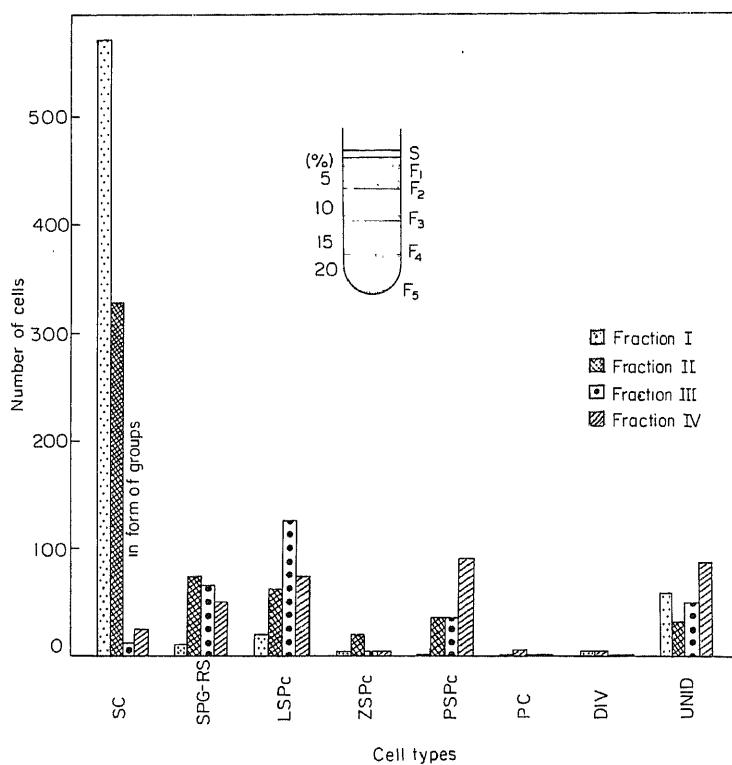
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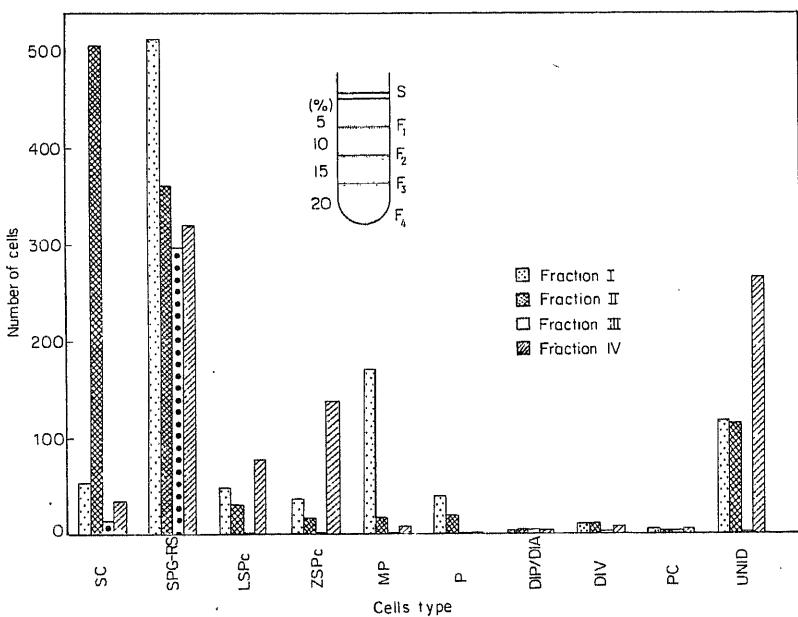
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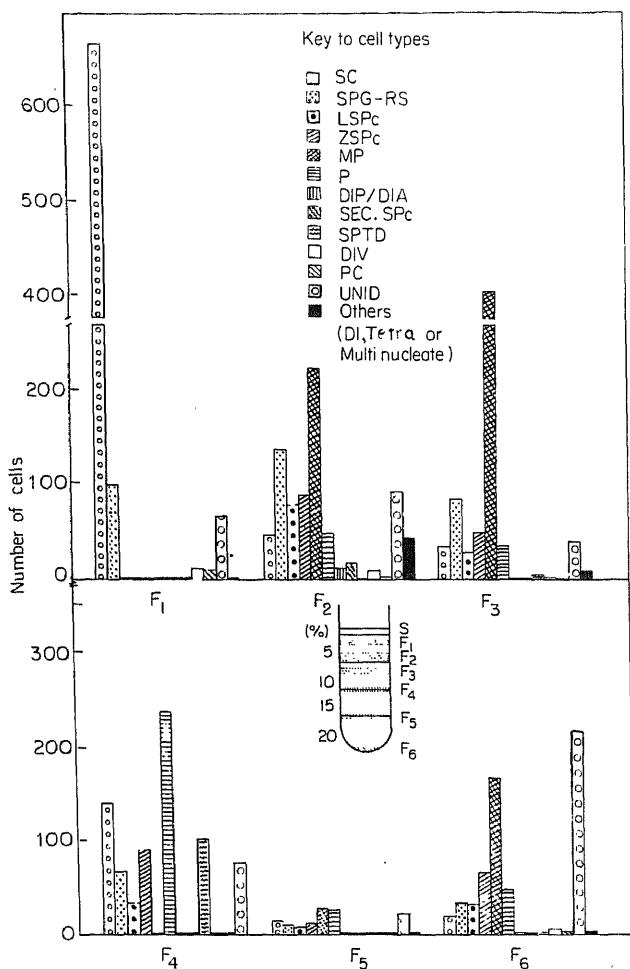
Figure 9.
Figures 5–9. Histograms of the distribution of specific cell types in the bands (F_1, F_2 etc) obtained by Ficoll gradient centrifugation of single cell suspensions. Details in the text. Inset in each figure is a diagrammatic representation of the banding pattern obtained. **5.** Day 8. **6.** Day 14. **7.** Day 18. **8.** Day 23. **9.** Day 30.

S, Sample layer on the gradient at the time of loading; PSC, presumptive Sertoli cells; SC, Sertoli cells; SPG, spermatogonia; SPG–RS, spermatogonia and preleptotene spermatocytes; LSPc, leptotene spermatocytes; ZSPc, zygotene spermatocytes; PSPc, pachytene spermatocytes; MP, mid-pachytene spermatocytes; P, late-pachytene spermatocytes; DIP/DIA, diplotene/diakinesis spermatocytes; SEC SPc, secondary spermatocytes; SPTD, spermatids; PC, peritubular cells; S.C.DIV., presumptive Sertoli cells in division; G.C.DIV./DIV., germ cells in division; UNID, unidentified cells; Others, di-, tetra- or multi-nucleate germ cells.

was rapidly passed through the hypodermic syringe. The resultant supernatant contained mostly blood cells and the perivascular-Leydig cells and was discarded. It was found that incubating the seminiferous tubule fragments simultaneously with Collagenase and Pancreatin, rather than sequentially, was quite effective in freeing the SGAs of peritubular and Leydig cells and had the obvious advantage of saving time and an additional step.

Another significant departure from the Welsh–Wiebe procedure was the substitution by Tris of the phosphate buffer used in the enzyme incubation step so as to avoid precipitation of the added Ca^{2+} required for Collagenase activity (as the phosphate salt). Due note was taken of the temperature-dependence of the pH of Tris-HCl buffers.

The SGAs recovered following the enzyme treatment could be fairly readily converted to single cell suspensions. However, with material from the older donors —days 18, 23 and 30—a small population of SGAs (less than 5% of the total cell mass) persisted as aggregates, possibly due to the presence of tight junctions between adjacent Sertoli cells, known to appear in the rat between days 15 and 18 (Vitale *et al.*, 1973). Vortexing proved effective in disaggregating such SGAs, but with a drastic reduction in the viability of the single cells thus obtained.

In our hands, the technique of separating the cell types in the single cell suspensions on stepwise gradients of Ficoll-400 proved reliable and satisfactory (table 1). For reasons not readily apparent, the SC-fraction from day 23 animals was heavily contaminated with germ cells—mostly spermatogonial cells and early spermatocytes.

It is pertinent to point out that unlike the earlier published procedures which require a few hours/few days to effect the needed separation, the method described here requires no more than 1·5 h to recover both Sertoli cells and germ cells from single cell suspensions, irrespective of the age of donor rats used in this study. Based on the criterion of exclusion of the vital dye, Trypan blue, the isolated single cell populations showed better than 95% viability. Furthermore, the isolated Sertoli cells could be successfully cultured to form monolayers and maintain viability for atleast 72 h.

Acknowledgement

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Plasma levels of gonadotropin releasing hormone during menstrual cycle of *Macaca radiata*

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Abstract. A sensitive radioimmunoassay for gonadotropin releasing hormone has been developed. The assay has been validated for its specificity by testing various analogues of gonadotropin releasing hormone. Analysis of plasma samples during the menstrual cycle of 4 female bonnet monkeys showed a significant increase in the immunoreactive gonadotropin releasing hormone levels during preovulatory period of the menstrual cycle.

Keywords. Gonadotropin releasing hormone; radioimmunoassay; monkey; menstrual cycle.

Introduction

Several recent studies have indicated the presence of gonadotropin releasing hormone (GnRH) like material in the placenta of human (Khodr *et al.*, 1980; Tan and Rousseau, 1982; Seeberg and Adelman, 1984), rat (Gautron *et al.*, 1981) and rabbit (Nowak *et al.*, 1984). It has also been suggested that placental GnRH has an important role in regulation of chorionic gonadotropin secretion (Khodr and Siler Khodr, 1978, 1986; Ashitaka *et al.*, 1980; Rao *et al.*, 1984a). Several of the above cited studies have been carried out using human placental explants under *in vitro* conditions. Recently, we have demonstrated (Rao and Moudgal, 1984b) that injection of GnRH by intravenous route to pregnant monkeys during early stages results in a significant increase in serum chorionic gonadotropin (CG) levels, thus providing first *in vivo* evidence for a role for GnRH in regulation of CG secretion in primates. Although several reports are available on the plasma levels of GnRH during menstrual cycle of the human female, practically no information is available on the GnRH levels during menstrual cycle or pregnancy in non-human primates. In view of this and as an extension of our *in vivo* study, we have initiated detailed studies on the role of GnRH during menstrual cycle and early pregnancy in bonnet monkeys. The present paper describes the development and application of radioimmunoassay (RIA) of GnRH to monitor the plasma levels of GnRH during menstrual cycle of bonnet monkeys, *Macaca radiata*.

Materials and methods

GnRH was obtained from Ayerst and Wyeth laboratories, New York, USA; other analogous used in the study were gifts from the following sources: Buserelin from Dr. J. Sandow, Hoechst, Federal Republic of Germany; GnRH-proethylamide, D-Trp^6-GnRH and D-Lys^6-GnRH from NIH, USA; D-Phe^6-GnRH, Trp^7-Leu^8-GnRH and D-Phe^6-Trp^7-Leu^8-GnRH from Dr. G. Keri, Hungary. Antiserum to GnRH was raised in adult male rabbits according to the procedure of Koch *et al.* (1973). The antiserum was characterized by checking its binding to iodinated GnRH and its cross reactivity with several analogues of GnRH. Iodination of GnRH was done using Iodogen method. The separation of iodinated GnRH from free iodine was achieved by fractionating the reaction mixture on G–25 Sephadex column (45 × 0·9 cm) equilibrated with 0·01 M phosphate buffered saline containing 0·1% gelatin-phosphate buffered saline 0·01 M, pH 7·4 (GPBS). The specific activity of iodinated GnRH ranged from 200–250 μCi/μmol. All other reagents used in the study were analytical grade, procured from local sources.

RIA of GnRH

Varying quantities of GnRH (1–500 pg in 100 μl) were added to 3·0 ml glass tubes containing 100 μl of rabbit antiserum to GnRH (final dilution of 1:3,000) followed by 100 μl of [^{125}I]-GnRH (30,000 cpm). The total incubation volume was 300 μl and the incubation was carried out at 4°C for 12 h. Separation of the free label from bound was achieved according to the method of Hichens *et al.* (1974) by addition of 500 μl of Dextran coated charcoal (0·3% dextran T 70 and 3% activated charcoal in 0·01 M PBS, pH 7·4) followed by centrifugation at 3000 *g* for 15 min at 4°C. Four hundred μl aliquot of the supernatant was counted in a LKB gamma counter.

Animals

Regularly cycling adult female monkeys weighing 5–6 kg were used in the study. The husbandary of the monkey colony was as described earlier (Prahlada *et al.*, 1975). The day on which vaginal bleeding was first seen was considered as day one of the menstrual cycle. The average length of the menstrual cycle of monkeys in the colony is 28 ± 2 days (n = 30). Blood samples were collected from unanesthetised animals between 11.00–11.30 a.m. on every day using heparinised vaccutainer tubes and plasma was separated by centrifuging at 1000 *g* for 5 min at room temperature.

Extraction of plasma

The clear plasma (about 1·5–2·5 ml) was transferred to tubes containing 10·0 ml of methanol, vortexed for 2 min and centrifuged at 10,000 *g* for 5 min. The supernatant was saved and the pellet was re-extracted with 10·0 ml methanol for 2 min and the two supernatants were pooled and evaporated to dryness at 55°C in a waterbath. The residue was reconstituted in 0·5 ml of GPBS and suitable aliquots were used for the assay.

Recovery of GnRH during extraction was ascertained by following the recovery of added [^{125}I]-GnRH. The recovery ranged from 78–84% (n = 9).

Validation of the GnRH assay

The RIA of GnRH was validated by assaying increasing quantities of plasma extract in the assay and checking for parallelism in the displacement curve obtained. The procedure employed to extract GnRH from the plasma was validated by monitoring the increase in GnRH levels following a bolus injection of GnRH. Two male monkeys were injected with a dose of 100 μg GnRH by intravenous route *via* femoral vein and blood samples were collected at specified intervals and the plasma obtained were extracted as described earlier and assayed for GnRH, following reconstitution.

Results

A standard inhibition plot for GnRH is presented in figure 1 and it can be seen that the assay range is from 10–250 pg of GnRH. The lowest concentration of GnRH which can be detected with certainty was 15 pg. The antiserum seems to be highly specific for the native GnRH sequence as all analogues tested showed less than 1% cross reactivity at 50% displacement of the assay. Plasma used for parallel displacement was a concentrated extract of plasma from female monkeys during various phases of menstrual cycle. The extracted plasma showed a parallel displacement curve (figure 1) in the GnRH-RIA similar to that of standard. The intra and inter assay variations were 8·5% and 10·8%, respectively.
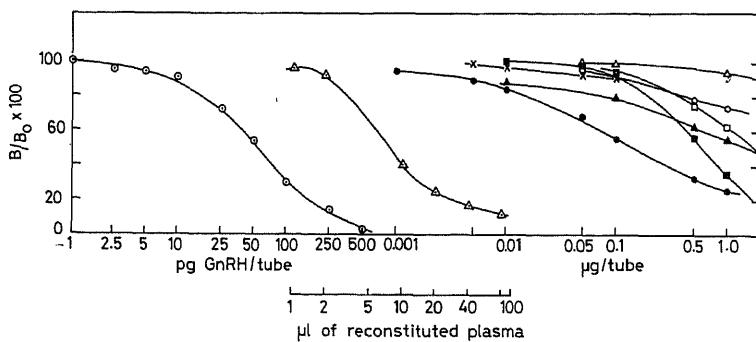
Figure 1. Standard inhibition plot for GnRH-RIA. ($\odot$), Cross reaction for analogues of GnRH; ($\bullet$), Buserelin; ($\square$), D-Trp^6-GnRH; ($\blacktriangle$), D-Lys^6-GnRH; ($\circ$), D-Phe^6-GnRH; ($\triangle$), Trp^7-Leu^8-GnRH; ($\times$), D-Phe^6-Trp^7-Leu^8-GnRH; ($\blacksquare$), GnRH-proethylamide; ($\triangle$), parallel displacement by plasma extract.

Validation of the GnRH-RIA

The results presented in figure 2 clearly show that following injection of a bolus dose of GnRH, there is a significant increase in the plasma levels of GnRH. The preinjection values are 5–8 pg in monkey No. 456 and 10–20 pg in monkey No. 726 and following injection of GnRH, by 5 min the plasma value increased 5–6 fold from 0 min value in both monkeys, indicating that only GnRH is quantitated by RIA and the observed GnRH in RIA is not due to any non-specific interference.
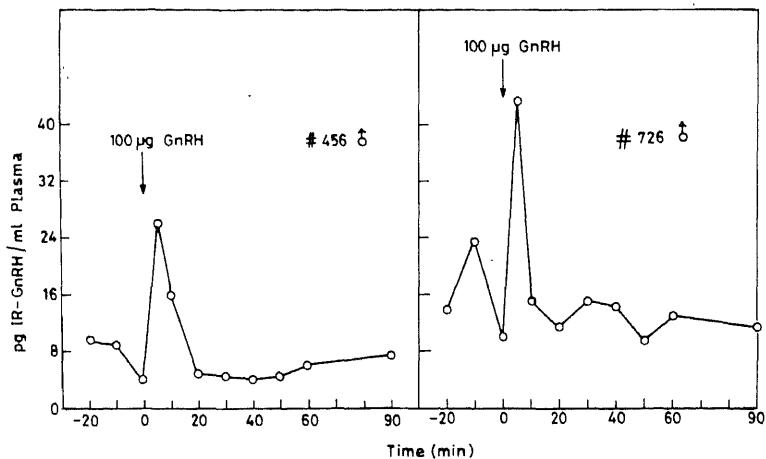
Figure 2. Plasma immunoreactive GnRH levels in adult male monkeys following injection of 100 μg of GnRH by intravenous route.

The increase observed however does not account for all the GnRH injected and it is known that more than 80% of the injected GnRH is cleared from the plasma within 2 min (Elkind-Hirsch *et al.*, 1982) in the case of humans. It can also be seen that the values returned to basal levels by 10 min and the fall in plasma levels is very rapid indicating that GnRH is cleared very fast in monkeys as in the case of humans.

Plasma GnRH levels during menstrual cycle in the monkey

The profiles of plasma immunoreactive GnRH levels during the menstrual cycle in the 4 female bonnet monkeys is shown in figure 3. The levels ranged from
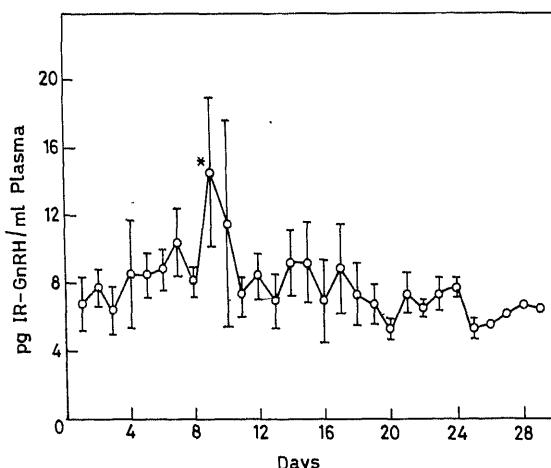
Figure 3. Plasma immunoreactive levels of GnRH during menstrual cycle in adult female monkeys. Mean ± SEM, n = 4
*Significantly different from day 1 value $P < 0.05$.

7–10 pg/ml during follicular phase, following which a significant ($P<0.05$) elevation of immunoreactive GnRH was seen by 9th day and the level decreased to basal levels by 11th day after which there was no significant variation.

Discussion

In the present study a specific RIA has been developed for monitoring plasma GnRH levels. The RIA has been validated for specificity and by monitoring the levels of GnRH following a bolus injection of GnRH, we have established the authenticity of the GnRH quantitated by RIA. The endogenous immunoreactive GnRH appear to be indistinguishable from the synthetic decapeptide used as a standard and the inhibition plot obtained by using various quantities of plasma extract was completely parallel to the standard curve. However, the discrepancy in the quantity of injected GnRH and the peak values obtained could be due to the interval in sampling. It may be pertinent to point out that in the studies of Elkind-Hirsch *et al.* (1982) the maximum values obtained at 1 min sampling was 515 pg, following 100 μg of GnRH injection and this also does not account for all the GnRH injected.

The observation that peak immunoreactive GnRH levels occur by 9th day of menstrual cycle in the bonnet monkey is in agreement with the report of Elkind-Hirsch *et al.* (1982) that peak levels of immunoreactive GnRH occur during preovulatory phase of human menstrual cycle. It is known that in the bonnet monkey serum estradiol–17 β surge occurs around day 9–10 and preovulatory surge of lutinising hormone (LH) occurs within 24 h of this surge (Rao *et al.*, 1984c). In this connection, the observation made in the present study, that the plasma GnRH also reaches peak value in bonnet monkeys by 10th day of menstrual cycle is of considerable significance and interest. It is possible that this increase in GnRH in levels may also have a role in the regulation of the release of gonadotropins during preovulatory surge in non-human primates also (Crowley *et al.*, 1985).

Acknowledgements

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Gonadotropin releasing hormone in first trimester human placenta: Isolation, partial characterisation and *in vitro* biosynthesis

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Abstract. Using a specific radioimmunoassay for gonadotropin releasing hormone, the presence of gonadotropin releasing hormone like material in the first trimester human placenta has been demonstrated. The material has been partially characterized using carboxy methyl cellulose chromatography, high pressure gel permeation chromatography and reverse phase C18 high pressure liquid chromatographic analysis. Analysis for bioactivity revealed that placental gonadotropin releasing hormone is much more active than synthetic gonadotropin releasing hormone in *in vitro* rat pituitary lutinising hormone release assay. *In vitro* biosynthetic studies using labelled precursors and immunoaffinity chromatography indicated that first trimester human placenta synthesizes gonadotropin releasing hormone like material.

Keywords. Placenta; gonadotropin releasing hormone; receptor assay; biosynthesis; immunoaffinity.

Introduction

The presence of extra hypothalamic gonadotropin releasing hormone (GnRH) like material has been reported in placenta (Gibbons *et al.*, 1975; Khodr *et al.*, 1980; Nowak *et al.*, 1984), testes (Hedger *et al.*, 1985), ovary (Aten *et al.*, 1986) and adrenal cortex (Gautron *et al.*, 1981). Though the precise role of the extra hypothalamic GnRH is not well established, it has been suggested that this exerts its action locally, as an autocrine system (Hsuesh *et al.*, 1982). Thus several studies carried out using human term placenta have suggested that placental GnRH has an important role in the regulation of chorionic gonadotropin (CG) secretion by placenta (Khodr *et al.*, 1978; Siler–Khodr *et al.*, 1986; Rao *et al.*, 1984; Das *et al.*, 1983; Malik *et al.*, 1986). However, it is known that human term placenta is minimally active as far as CG production is concerned and placental CG production is maximal during the first 6–8 weeks of pregnancy. Thus studies using placenta from early stages can be more meaningful and recently we have demonstrated that GnRH exerts a differential effect (Rao *et al.*, 1985) on CG production during short term incubation of first trimester human placental minces. This observation as well

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as the fact that so far only term human placental GnRH has been well characterised (Tan *et al.*, 1982; Seeberg *et al.*, 1984) prompted us to look for the presence and biosynthesis of GnRH like material in the first trimester human placenta.

Materials and methods

Synthetic GnRH was obtained as a gift from Ayerst and Wyeth laboratories, New York, USA. The source of various analogues used in the study, the development and validation of radioimmunoassay (RIA) of GnRH used in following the purification of placental GnRH have been described in the preceeding paper (Mathialagan *et al.*, 1986). Antiserum to GnRH raised in goat, used in the immunoaffinity column, was generously provided by Dr. H. M. Fraser, MRC Reproductive Biology Unit, Edinburgh, UK. Coupling of anti GnRH immunoglobulin G was done according to the procedure given by the suppliers (Pharmacia, Sweden). Carboxy methyl cellulose (CM cellulose), cyanogen bromide activated sepharose 4B, cycloheximide and bovine serum albumin (BSA) were obtained from Sigma Chemicals Co., St. Louis, Missouri, USA. [^{3}H]-Leucine (specific activity 100 Ci/mmol) and [^{125}I]-sodium iodide were obtained from Amersham International, England. [^{14}C]-Chlorella hydrolysate (specific activity 20 mCi/m atom C) was supplied by Bhabha Atomic Research Centre, Bombay. Eagle's minimum essential medium (EMEM) was purchased from Gibco Laboratories, New York, USA. Earle's balanced salt solution (EBSS) was obtained from Hi Media, Bombay. Highly purified hCG (CR-123, 12,000 IU/mg) used for iodination was generously provided by NIAMDD, USA. All other chemicals used in the study were of analytical grade procured from local sources.

Collection of human placenta

Placenta was collected from cases of medical termination of pregnancy (6–10 weeks) from local hospital. The placenta along with blood was collected in EBSS and quickly transported on ice to laboratory. After decanting the blood, placenta was extensively washed with EBSS.

Extraction and fractionation of GnRH

Placental villi were minced and processed essentially according to the procedure of Guillemin *et al.* (1963). The minces were homogenized with 2 N acetic acid and the homogenate was spun at 10,000 g for 20 min at 4°C. The clear supernatant was lyophilized (fraction A) and stored at −20°C until further processing. Fraction A was reconstituted (5 mg protein/ml) in distilled water and loaded on CM cellulose column (10 × 1·5 cm) equilibrated with 0·01 M ammonium acetate, pH 4·5. Flow rate was maintained at 8 ml/h to ensure complete retention of GnRH like material on to the column. The column was washed extensively with equilibration buffer until there was no detectable absorbance at 280 nm, in flow through fractions. Elution was carried out with 0·1 M ammonium acetate, pH 7·5 and 2·0 ml fractions were collected. The optical density was monitored at 280 nm and immunoreactivity in each fraction was assayed by GnRH specific RIA. The fractions which showed

immunoreactivity and eluted at a position corresponding to standard GnRH were pooled and lyophilized (fraction B).

High pressure liquid chromatographic analysis

High pressure gel permeation chromatography (HP-GPC) of fraction B was carried out on a TSK G2000 SW ultrapac column (LKB, 7.5×600 mm, Fr. range 0.5–60 K). The chromotography system was equipped with two pumps, a solvent programmer attached to UV absorbance detector set at 226 and 280 nm. The equilibration buffer was 0.05 M phosphate buffer pH 6.8 containing 0.1 M NaCl. The column was calibrated with standard molecular weight markers (GnRH 1.1 K, ACTH 4.5 K, β-LPH 9.8 K, Cyt. C 12.4 K, RNase 13.7 K, trypsin inhibitor 20 K, ovalbumin 44 K and BSA 65 K). Synthetic GnRH eluted at an elution volume of 35 ml and fractions collected were monitored at 226 and 280 nm and also for immunoreactivity by GnRH specific RIA.

Fraction B was also subjected to reverse phase high pressure liquid chromatography (HPLC) analysis using C18 column according to the procedure described earlier (Nikolics *et al.*, 1983). The isocratic system used in the analysis consisted of 58% (v/v) ammonium acetate. 0.05 M and 42% (v/v) methanol (pH 4.2). Synthetic GnRH had a retention time of 13 min in this system. Gradient elution was carried out using the following solvents: (A) 90% (v/v) methanol and 10% (v/v) phosphate buffer; 0.1 M, pH 2.0, (B) 10% (v/v) methanol and 90% (v/v) phosphate buffer, 0.1 M, pH 2.0. In the gradient elution synthetic GnRH eluted with a retention time of 22.5 min.

Bioactivity of placental GnRH

The biological activity of placental GnRH was determined using the *in vitro* rat pituitary lutinising hormone (LH) release bioassay as described by Sairam *et al.* (1981). Pituitaries from 20 day old Wistar male rats were incubated at 37°C with varying concentrations of GnRH (1–10 ng/ml) in EMEM for 4 h under 95% O_2 and 5% CO_2. Medium was separated by centrifugation at 3000 g for 20 min at 4°C and analysed for LH by human chorionic gonadotropin (hCG) radioreceptor assay as described by Sairam *et al.* (1981). Iodination of highly purified hCG was done according to the method of Greenwood *et al.* (1963) and the specific activity ranged from 40–50 μCi/μg. The assay range was from 0.5–50 ng/tube and minimum detectable quantity of rat LH equivalent was 1 ng/tube. The inter and intra assay variation of the assay were 12.4% and 9.5%, respectively.

In vitro biosynthesis of GnRH

Placental minces were incubated with 100 μCi of [^{14}C]-chlorella protein hydrolysate or [^{3}H]–leucine in the presence of 0.01% bacitracin in 1 ml of EBSS for 2 h at 37°C under 95% O_2 and 5% CO_2 in a Dubnoff shaker. Where appropriate, cycloheximide was used at a concentration of 500 μM. After the incubation, 5 volumes of 2 N acetic acid were added to the incubation flask and the minces were homogenized using ground glass homogenizer. The homogenate was centrifuged in cold at 10,000 g and the clear supernatant was lyophilized and fractionated on CM cellulose column as described earlier. The radioactive fractions which eluted at a

position corresponding to that of synthetic GnRH were loaded on a column of anti GnRH IgG coupled to cyanogen bromide (CNBr) activated sepharose. Equilibration and washing of the immunoaffinity column was performed using 0·01 M phosphate buffer pH 7·4 containing 0·9% (v/v) NaCl. GnRH was eluted from the column using 1 M acetic acid (Akanxuma *et al.*, 1974). Fractions were monitored for radioactivity and absorption at 280 nm. In the experiment where [^{3}H]-leucine was used as a precursor approximately 25,000 cpm and in case of [^{14}C]-chlorella hydrolysate, approximately 4,00,000 cpm were loaded on the affinity column. In both cases recovery of the radioactivity was 90–95% with about 3% of the radioactivity being specifically eluted with 1 M acetic acid.

Results

Detection of GnRH like material in placenta by RIA

In order to ascertain the presence of GnRH like material, 2 N acetic acid extract (fraction A) of the first trimester human placenta was assayed in the GnRH specific RIA. It can be seen from the data presented in figure 1 that fraction A gave a dose dependent displacement. In acetic acid extraction and CM cellulose chromatography of homogenate caused significant enrichment of placental GnRH like activity (table 1).
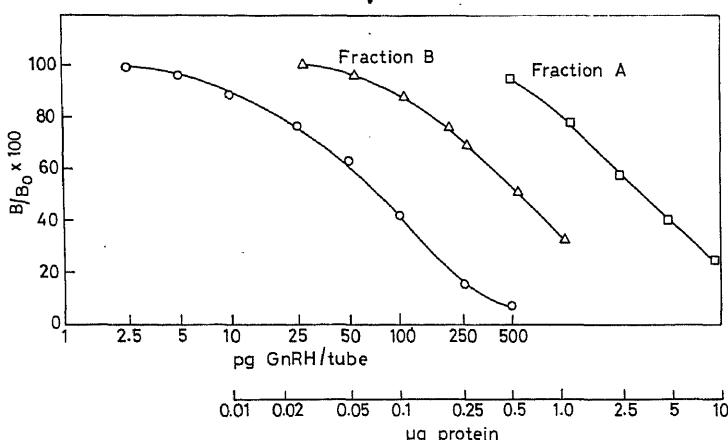
Figure 1. Standard inhibition plot for GnRH; parallel displacement by fraction A and B.

CM cellulose chromatography

The specific elution profile of synthetic GnRH on CM cellulose column is shown in figure 2A. This elution profile is characteristic of synthetic GnRH (Guillemin *et al.*, 1963) and is highly reproducible under standardised conditions. It can be seen that following fractionation on CM cellulose column, fraction A gave a peak whose elution pattern was exactly similar to that of standard GnRH (fraction B). Analysis of the fractions from this peak for immunoreactivity by GnRH RIA (figures 1 and

Table 1. Purification of placental GnRH.

Fraction	Qty. of immunoreactive GnRH/mg protein	Fold purification
Homogenate	116·6 pg	1
2 N Acetic acid extraction (fraction A)	21·0 ng	180
CM cellulose chromatography (fraction B)	123·0 ng	1054
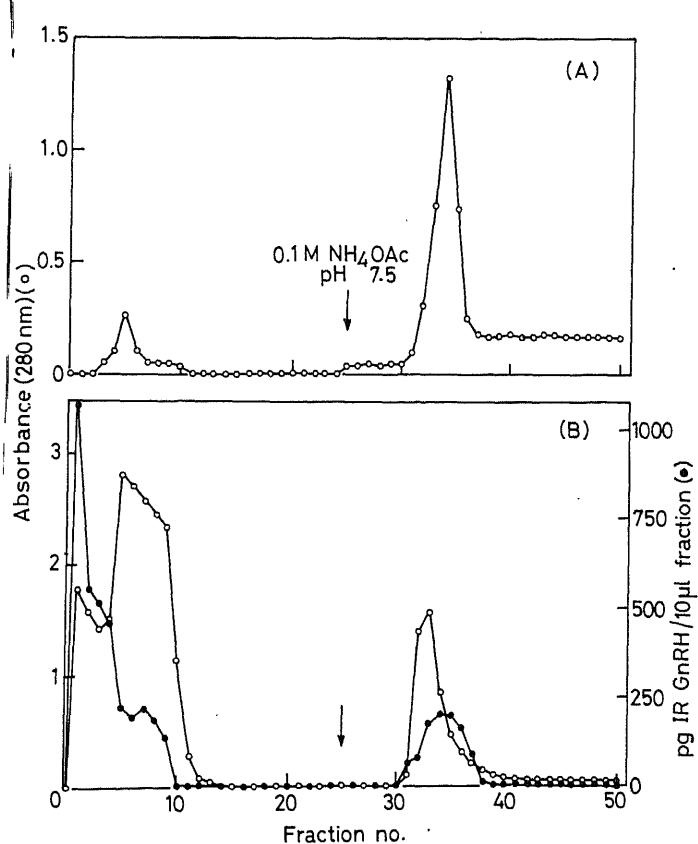
Figure 2. Elution profile on CM cellulose column. The column (1·5 × 7 cm) was equilibrated with 0·01 M ammonium acetate, pH 4·5. Elution was done with 0·1 M ammonium acetate, pH 7·5. (A), Synthetic GnRH; (B), fraction A.

2) indicated that there is a significant immunoreactivity associated with this peak. It should be pointed out that fraction A gave in addition to the peak corresponding to GnRH, another peak which did not bind to the column with which considerable immunoreactivity is also associated. However, in view of the fact that elution

profile of synthetic GnRH is a very characteristic feature, only fraction B was subjected to HPLC analysis.

HPLC analysis

The profile obtained following analysis of fraction B on HP-GPC is shown in figure 3. It can be seen that the fraction B showed an absorption peak (figure 3B) at an elution volume of 35 ml which corresponded well with the immunoreactivity (figure 3C) as well as standard elution profile of GnRH (figure 3A). As in the case of CM cellulose analysis, a peak of immunoreactivity in the fractions which eluted very early (elution volume less than 8 ml) was noticeable. The results of analysis of fraction B on reverse phase C18 (RP C18) column using isocratic system and gradient elution are shown in figures 4 and 5, respectively. It can be seen that in the isocratic system, a clear peak corresponding exactly to the retention time 13 min of standard GnRH was observed. In the case of gradient elution (figure 5) a peak corresponding to the retention time 22·5 min of standard GnRH was noticed and it can also be seen that the peak heights were concentration dependent. All these results together strongly
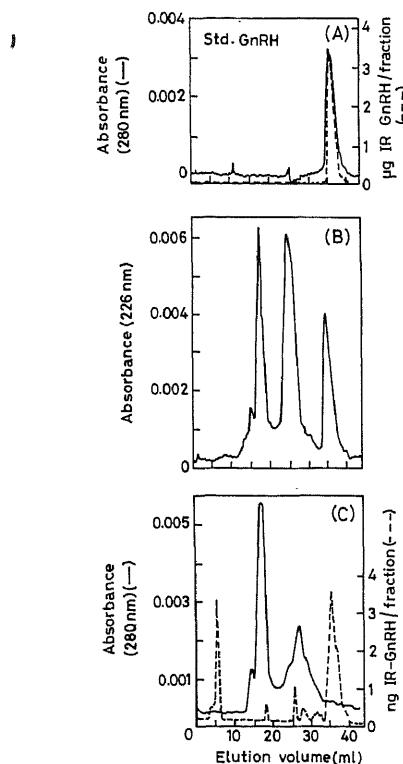
Figure 3. HPGPC analysis of fraction B. See methods for details of column and buffer used. Elution profile of (A) standard GnRH, (B) and (C) fraction B.
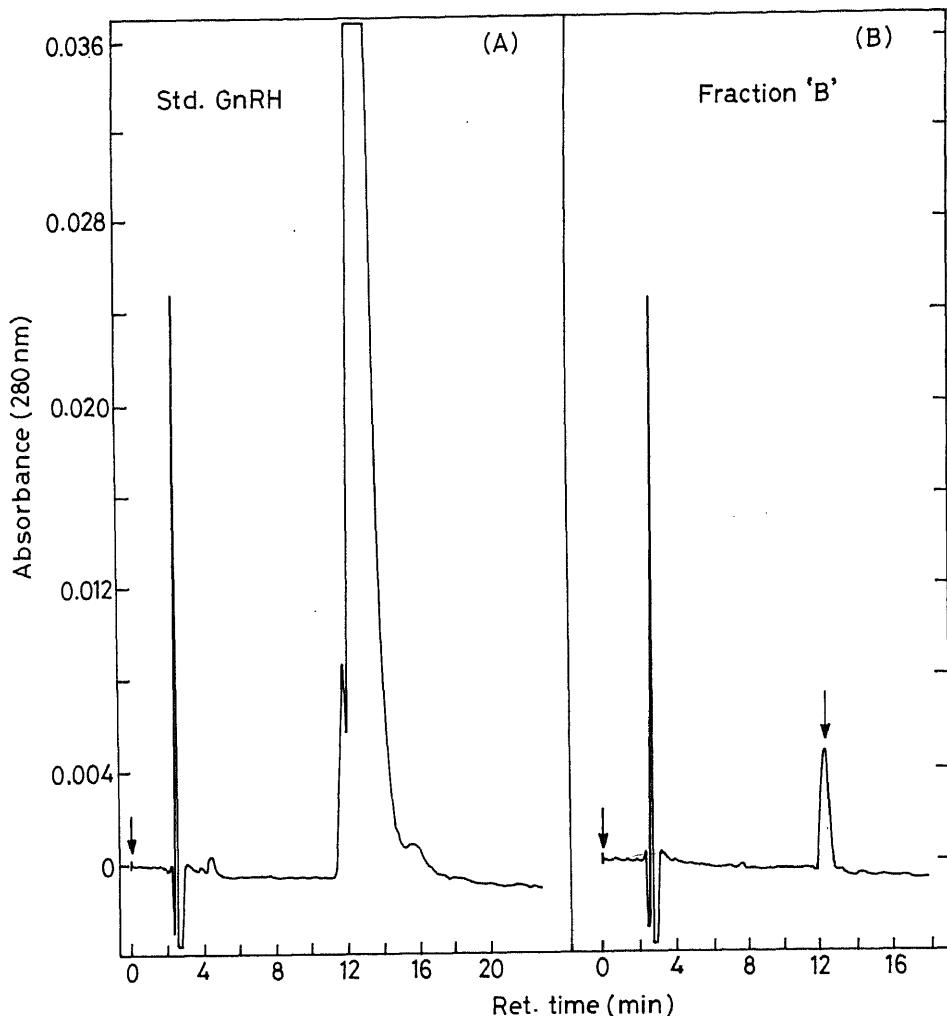
Figure 4. RP C18 HPLC analysis: Isocratic system.

support the conclusion that there is a material which is very similar to hypothalamic GnRH in the first trimester human placenta.

Biological activity of placental GnRH

The results presented in figure 6A show that following addition of 1–10 ng of synthetic GnRH there was a significant, dose dependent increase in the hCG equivalent of rat LH released into the medium, thus validating the radioreceptor assay. The results of the effect of addition of fraction A and fraction B on the release of LH into the medium is shown in figure 6B. It can be seen that both fractions showed significant, dose dependent stimulation. Interestingly both fractions exhibited much higher activity than standard GnRH. The highest quantity of
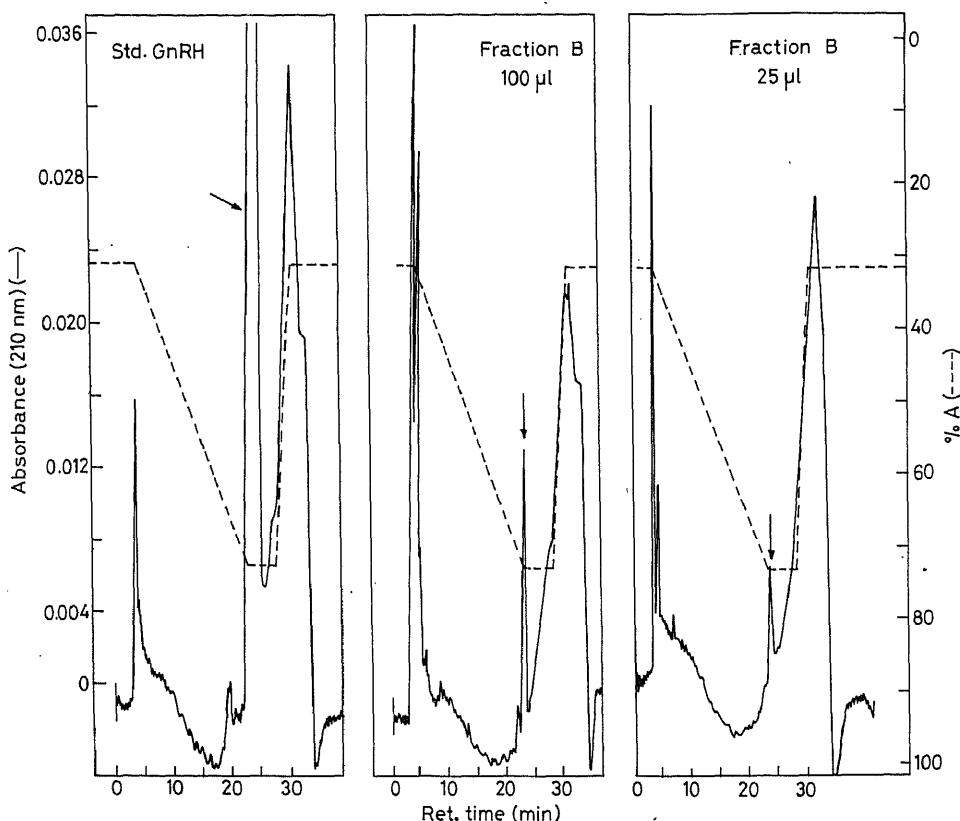
Figure 5. RP C18 HPLC analysis: Gradient elution.

fraction A and B tested corresponded to 0·5 ng and 0·25 ng of immunoreactive GnRH and at these concentrations, they were 3 and 4 fold more active respectively than synthetic GnRH used in the study.

In vitro biosynthesis of placental GnRH

Biosynthesis of placental GnRH was monitored by following the incorporation of [3H]-leucine or [14C]-chlorella hydrolysate into 2 N acetic acid extractable peptides which were fractionated on CM cellulose column. The results of this study are presented in figure 7. It is clear that the incorporation of both [14C] and [3H] radioactivity corresponding to the elution of standard GnRH on CM cellulose column could be observed. Analysis by specific RIA indicate that immunoreactivity is also associated with the radioactivity peak suggesting that the peak represents the newly synthesized GnRH. Experiments carried out in presence of cycloheximide (500 μm) indicate that the synthesis of GnRH is dependent upon protein synthesis as there is a considerable decrease in the incorporation of labelled precursor into this fraction (figure 8).
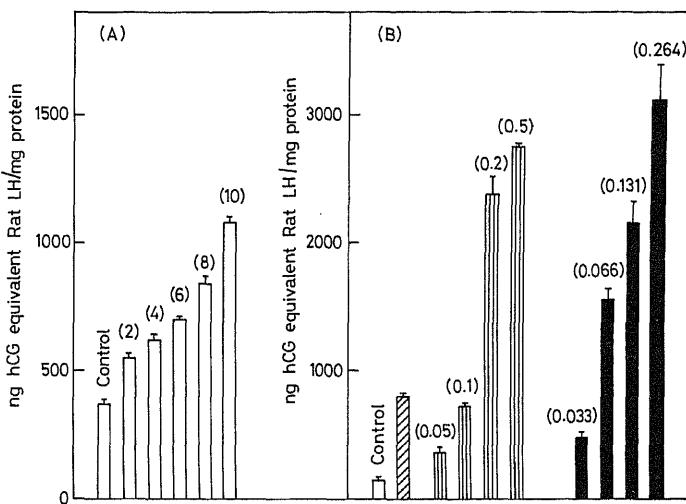
Figure 6. Bioactivity of placental GnRH. (A), Dose response with synthetic GnRH Quantity (ng) of GnRH added per tube indicated in parenthesis. (B) Bioactivity of (1), synthetic GnRH (10 ng/tube) ▨; (2), fraction A ▥; (3), fraction B ■. Figures in the parenthesis indicate the quantities (ng/tube) of immunoassayable GnRH added. Each point presents Mean ± SEM of triplicate determinations.

To further establish the identity of the radioactive peak corresponding to GnRH, this material was applied to GnRH immunoaffinity column and the results are presented in figure 9. It can be seen that in both experiments where [3H]-leucine or [14C]-chlorella hydrolysate was used as a precursor, a peak of radioactivity which corresponded to the elution profile of standard GnRH was noticeable, though the specific radioactivity recovered in the fraction was only about 3% of the total radioactivity applied to the column.

Discussion

The presence of GnRH like material in extrahypothalamic sources has been reported using immunochemical methods or the conventional isolation procedures or a combination of both (Khodr *et al.*, 1980; Gautron *et al.*, 1981; Tan *et al.*, 1982; Hedger *et al.*, 1985). We have been able to conclusively demonstrate the presence of GnRH like material in human placenta using a combination of immunological, biochemical and biosynthetic approaches. The RIA employed in the study has been validated for its specificity (Mathialagan *et al.*, 1986). As mentioned earlier the elution profile of hypothalamic GnRH on CM cellulose column was characteristic and we have successfully employed this in following the purification of placental GnRH. The purification achieved at various steps is also a clear indication that we have purified a GnRH like material. HPLC analysis provided an additional evidence for the identity of a placental GnRH like peptide. Most importantly the material isolated from placenta had biological activity similar to that of GnRH in the *in vitro* rat pituitary assay. We have also consistently observed that placental
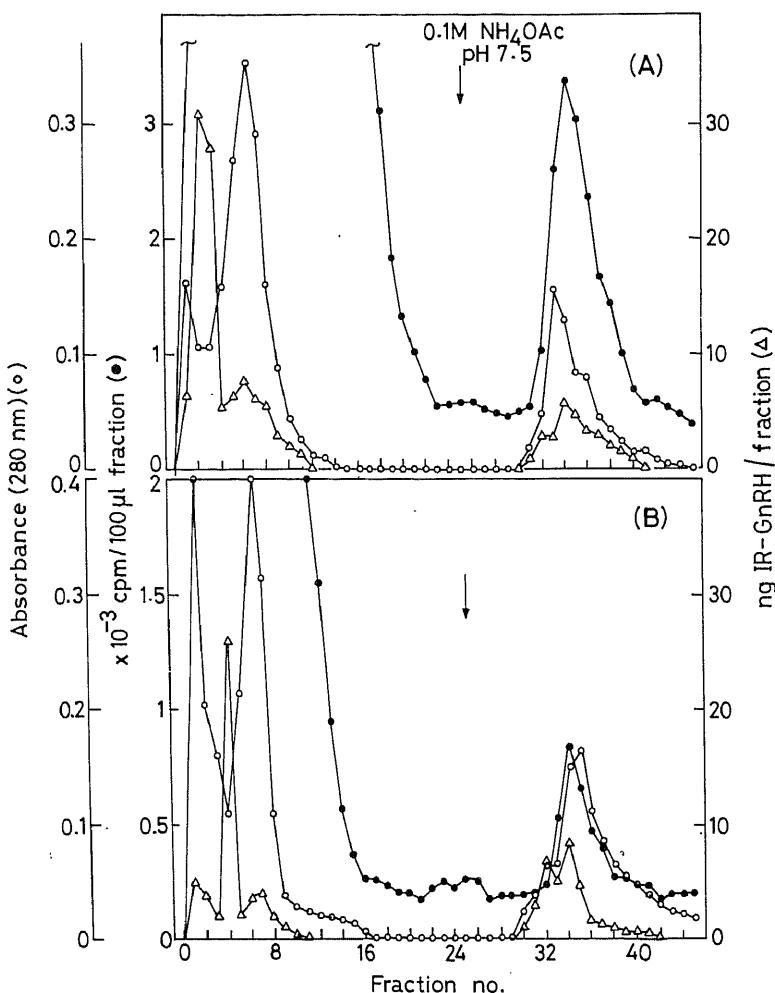
Figure 7. *In vitro* biosynthesis of placental GnRH: Elution profile on CM cellulose column of 2 N acetic acid extractable peptides. (A), [^{14}C]-chlorella hydrolysate; (B), [^{3}H]-leucine.

GnRH like material was found to be more active than synthetic GnRH in the bioassay and interestingly a similar observation has been made by Siler–Khodr *et al.* (1986). However, as the material has not been purified to homogenity, it is possible that the placental material may have in addition to native GnRH other materials which possess GnRH like activities. This conclusion is supported by the fact that HP-GPC analysis reveals multiple immunoreactive peaks in addition to the peak corresponding to standard GnRH (figure 3C). Recently Nowak *et al.* (1984) have suggested that the presence of significant part of GnRH like activity is due to factors other than GnRH in rabbit placenta.

It is known that the proteolysis of GnRH in tissues occurs between Gly^6-Leu^7 bond (Koch *et al.*, 1974) and the half life of GnRH is extremely low (Elkind–Hirsch
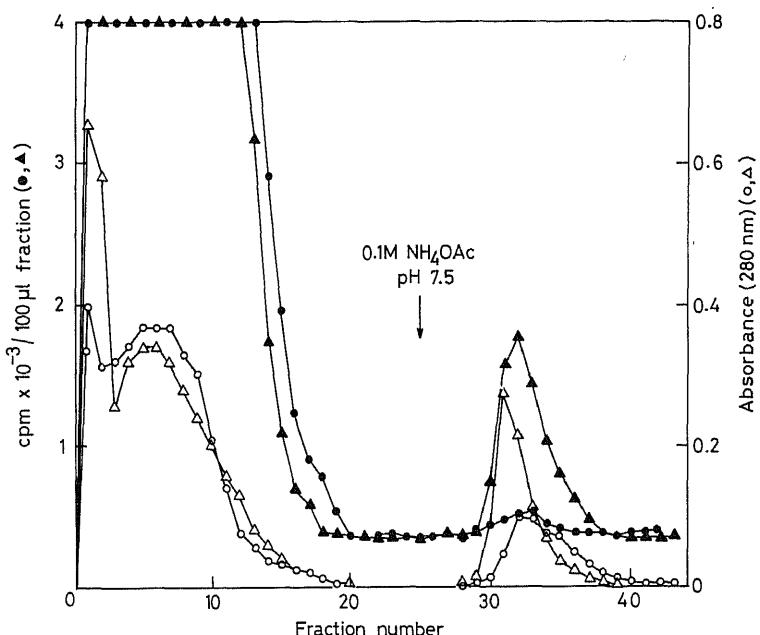
Figure 8. *In vitro* biosynthesis of placental GnRH: Effect of cycloheximide, 500 μM (○, ●).

et al., 1982). Thus use of [^{3}H]-leucine alone as a precursor in *in vitro* biosynthetic studies of GnRH will result in a decreased incorporation of label. In order to eliminate this uncertainty we have employed [^{14}C]-chlorella protein hydrolysate also as a precursor. Studies using immunoaffinity chromatography also support the conclusion that GnRH is biosynthesized in the first trimester human placenta. The immunological identity of the synthesized GnRH is further established by the fact that we have employed a rabbit antiserum to GnRH in RIA and a goat antiserum to GnRH for immunoaffinity chromatography. The foregoing results establish that placental GnRH also has similar immunological and biological characteristics of the hypothalamic counterpart.

During the course of CM cellulose analysis of fraction A we have consistently observed a peak of immunoreactive material in the flow through fractions. At the moment we are not clear as to the identity of this fraction. It may be pertinent to point out that post translational processing of the precursor of GnRH results into two peptide fragments (Nikolics *et al.*, 1985), one of which is a 56 amino acid C-terminal peptide (GnRH-associated peptide, GAP) having a prolactin release inhibiting activity as well as gonadotropin releasing activity in pituitary cell cultures. It may be possible that the immunoreactive CM cellulose flow through fraction represents the GAP peptide. Additional information as to the immunological reactivity of GAP with GnRH antiserum and its chromatographic fractionation will be of help in establishing the identity of the CM cellulose column flow through peptide observed in our studies.
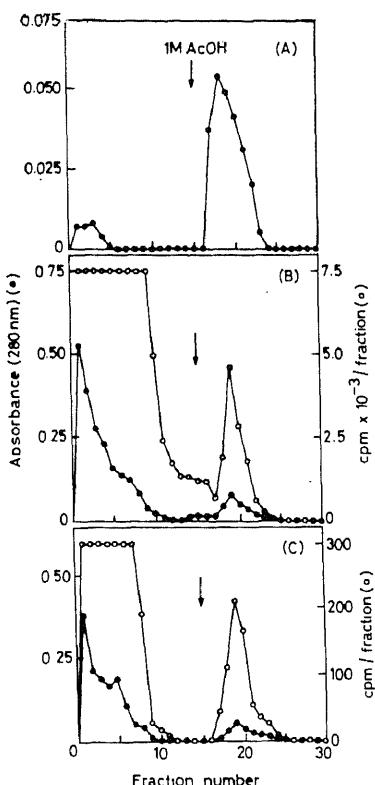
Figure 9. *In vitro* biosynthesis of placental GnRH: Immunoaffinity chromatographic analysis of fraction B. (A), Standard GnRH; (B), [^{14}C]-labelled fraction B; (C), [^{3}H]-labelled fraction B.

Acknowledgements

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Circadian and circannual changes in the testicular function of adult rhesus monkeys

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Abstract. The endocrine and gametogenic status of the testes were studied in 9 healthy adult rhesus monkeys of proven fertility throughout a one-year period. Testosterone levels were estimated by radioimmunoassay in blood samples collected at 4 h intervals over a 24 h period once a month. Semen samples and testicular biopsies were also examined once a month. A well-defined circandian rhythm was evident in serum levels of testosterone. The rhythmicity was less pronounced in February and September. The 24 h mean levels of serum testosterone were high between the months of August to March and low in the months of May to July. All animals did not uniformly respond to electro-ejaculation in April and May. Semen volume and total number of spermatozoa were maximal between September and March and least from April to August. Testicular biopsies indicated that all stages of spermatogenesis were evident between September and March and the spermatogenic activity was less evident between April and August. The contents of Sertoli cells showed a seasonal cyclicity; they were laden with lipid droplets during April to August when spermatogenesis was quiescent and vacuolated during September to March when spermatogenesis was active. These studies indicate that the testing of contraceptive drugs needs to be restricted to months of September to March in male rhesus monkeys otherwise, it is possible that the naturally occurring reproductive quiscence may be attributed to the effect of the drug being tested. The data accrued from the present studies also provide quantitative information on circulating levels of testosterone which could be used as a reference background while evaluating the contraceptive drug-effects in male rhesus monkeys.

Keywords. Testicular function; circadian and circannual changes; seasonality; rhesus monkey.

Introduction

New drugs developed for the regulation of human fertility are evaluated for their toxicological safety and efficacy in non-human primates because of the close similarities in their reproductive system to that of man (Diczfalusy and Standley, 1972; Prasad and Anand Kumar, 1977; Anand Kumar, 1980). The rhesus monkey is one of the most widely used species of non-human primates for such purposes. However, unlike man, the rhesus monkey exhibits a distinct seasonality in its reproductive performance (Prakash, 1962; Conaway and Sade, 1965; Southwick *et al.*, 1965; Vandenburg and Vessy, 1968; Michael and Keverne, 1971; Michael *et al.*, 1975; Gordon *et al.*, 1976; Anand Kumar *et al.*, 1980; Nieschlag and Wickings,

1980). These seasonal changes need to be taken into consideration while evaluating the effects of contraceptive drugs in the rhesus monkey.

This communication describes the seasonal changes occurring in serum levels of testosterone and the gametogenic function of the testes in a caged colony of rhesus monkeys. The information is of particular relevance to the preclinical evaluation of new fertility regulating drugs. As far as we are aware, this is the first description in which the endocrine function of the testis and its histological features are described in the same group of monkeys through the year.

Materials and methods

Animals

Nine healthy male monkeys of proven fertility (8–12 kg body weight) were selected from the breeding colony of the Primate Research Facility of the All India Institute of Medical Sciences. The animals were caged individually and maintained under uniform lighting schedule of 14 h (light):-10 h (dark) throughout the year. Animal husbandry methods followed in this facility have been described (Anand Kumar *et al.*, 1980).

Study design

This study was carried out between January and December. The animals were subjected to blood sampling, penile electro-ejaculation and testicular biopsies respectively on the 15th, 17th and 18th of each month.

Estimating circulating levels of hormones

Blood (10 ml per sample) was collected from conscious monkeys from the antecubital or saphenous vein once a month over a 24 h period at intervals of 4 h. Blood sampling was started at 08.00 h and completed at the same time on the following day. Thus, a total number of 7 blood samples was collected from each animal every month for 12 months. Serum was separated and stored at –20°C until levels of testosterone were estimated by radioimmunoassay (RIA) using reagents supplied by the WHO and following methods described in the WHO Method Manual (1981). The inter-and intra-assay variations were respectively 6·4 and 3·6% ($n=22$). The antisera for testosterone cross-reacted with dihydrotestosterone to an extent of 50% (Puri *et al.*, 1981). The data was subjected to logit-log transformation and statistical analysis was carried out by students 't' test (Snedecor and Cochrane, 1975) to determine the significance of differences between day and night levels of testosterone as well as between the 24 h mean testosterone levels during different months of the year.

Semenology

Monkeys were electro-ejaculated and the semen collected in clean vials. The total number of spermatozoa present in the ejaculate was determined as described by Belsey *et al.* (1980). The volume of semen was estimated by transferring the entire

ejaculate to a 10 ml graduated measuring cylinder containing 5 ml of distilled water and reading off the displaced volume of water.

Testicular biopsies

The testicular biopsies were performed at different sites in the following sequence: (i) the cranial end of the left testis, (ii) caudal end of the right testis, (iii) caudal end of the left testis and (iv) cranial end of the right testis. A few pieces of the tissue were immersed in Bouin's fluid, processed for paraffin embedding, sectioned at 6 μm thickness and stained with haematoxylin eosin. Other pieces of tissue were immersed in Karnovsky's fluid, processed, embedded in Araldite, sectioned at 1 μm thickness and stained with toludine blue.

Results

Testosterone levels

A well-defined circadian rhythm was evident in the circulating levels of testosterone (figure 1). The peak value of about 55 nmol·l^{-1} occurred around mid-night and the lowest value of about 17 nmol·l^{-1} occurred around mid-day. This circadian rhythmicity was evident during all the months of the year (figure 2). However, the magnitude of difference between the day and night levels was the least in February and September (figure 2).
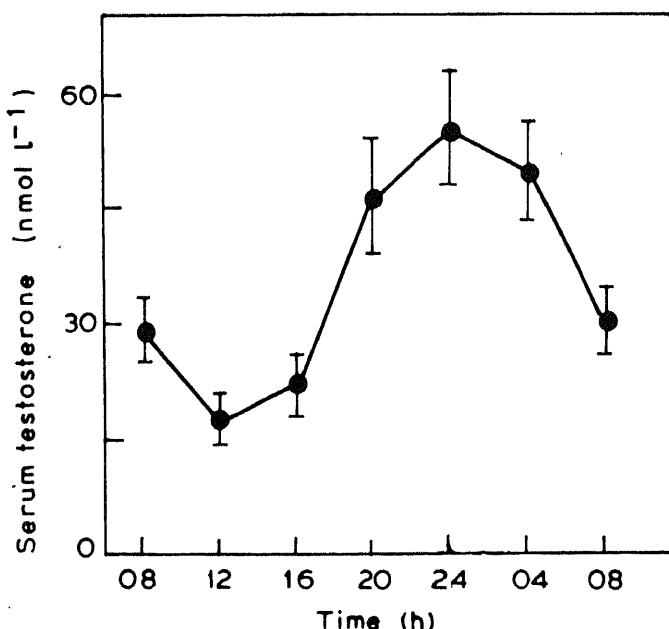
Figure 1. Serum testosterone levels at different times of the day in 9 monkeys studied throughout the year. The lowest level is seen around mid-day while highest level around mid-night. Each point represents the geometric mean (vertical bars indicate 95% confidence limits) of 108 observations.

Figure 2. Circadian pattern of circulating levels of serum testosterone in 9 monkeys studied throughout the year. The circadian rhythmicity is evident during all the months of the year. The magnitude of differences between day and night levels are least in February and September. Each point is the geometric mean of 9 observations with 95% confidence limits indicated in vertical bars.

A comparison of the mean values of testosterone levels in the pooled serum samples collected over a 24 h period showed that significantly high levels occurred during August, September and October and again in March. The values for these months were not significantly different from one another as they were for the other months.

Semenology

All the 9 animals responded positively to the electro–ejaculatory stimulus in January, the first month of the study. Thereafter, one monkey remained totally refractory to the stimulus throughout the year. The number of animals responding to electro–ejaculation also varied between the months (figure 3). The least number of responders were seen in April and May.

Seminal fluid volume was lowest from May to August and the volume began to rise from September onwards to reach maximal levels in November and remained so till February. Thereafter, the seminal fluid volume declined (figure 3). The total number of spermatozoa per ejaculate showed marked differences between individuals. However, mean values were lowest between the months of April to August and during the following months the number of spermatozoa increased to reach peak levels in November and remained so until January (figure 3).

Figure 3. Composite diagram to illustrate the 24 h mean levels of serum testosterone, semen volume and the total number of spermatozoa per ejaculate during different months of the year. Mean levels of serum testosterone for the months March, September and October are not significantly different from one another but are significantly ($P < 0.001$) different from the other months. Seminal volume is the lowest during May, June, July and August in contrast to the other months. The figures in parentheses indicate the number of animals out of the 9 monkeys studied responding to electro-ejaculation during each month. The total number of spermatozoa is the lowest during the months of April to August as compared with the remaining months.

Testicular histology

Marked seasonal changes were evident in the spermatogenic activity through-out the year. The seminiferous tubules were large and exhibited all stages of spermatogenesis between September and March. Between April and August there was an obvious reduction in the spermatogenic stages and mitotic divisions (figure 4).

Figure 2. Circadian pattern of circulating levels of serum testosterone in 9 monkeys studied throughout the year. The circadian rhythmicity is evident during all the months of the year. The magnitude of differences between day and night levels are least in February and September. Each point is the geometric mean of 9 observations with 95% confidence limits indicated in vertical bars.

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Figure 4. Photomicrographs of testicular biopsies taken in January (A), April (B), July (C) and October (D) and sectioned and stained with haematoxylin-eosin. Spermatogenic activity is low during April (× 320).

Araldite sections of testicular tissues stained with toludine blue showed the presence of metachromatically stained lipid droplets in the Sertoli cells. The Sertoli cells were fully laden with such droplets from April to August when spermatogenesis was quiscent. During the remaining months, when spermatogenesis was active, the Sertoli cells were depleted of their lipid droplet contents and only cytoplasmic vacuoles were evident (figure 5). Thus, the depletion of lipid droplets in the Sertolic cells was coincidental with the increase in spermatogenic activity.

Discussion

The present studies on a group of proven fertile males kept under captivity confirm earlier reports (Goodman *et al.*, 1974; Michael *et al.*, 1974, Perachio *et al.*, 1977) of the occurrence of a distinct circadian rhythmic pattern in the circulating levels of testosterone in rhesus monkeys. Peak levels occurred around mid-night and the lowest levels during mid-day. Our studies have also shown that although the circadian rhythmic pattern was evident throughout the year, the magnitude of difference between the peak and low levels was less evident during February and September as compared with the other months. This may well be due to variation between individual in which the actual peak levels occurred between 20.00 h and 04.00 h. It may be noted that February marks the end of winter in Delhi and the onset of spring while September marks the begining of autumn. It would be interesting to determine whether the marked individual variations observed in the time at which actual peak levels of testosterone occur during the night is indicative of the diurnal rhythmically undergoing re-entrainment in February and September which marks the begining of summer and winter.

Circadian rhythmicity in circulating levels of testosterone has been reported in a number of primate species including man. In man, peak levels of testosterone occur around 04.00 h (Barberia *et al.*, 1973) or 06.00 h (Sjorberg *et al.*, 1979). The biological significance of such circadian variations in circulating levels of testosterone remains to be determined. There is some evidence, however, to indicate that the abolition of the nocturnal surge of testosterone in the monkey may impair spermatogenesis (Moudgal *et al.*, 1985).

A comparison of the mean values of testosterone observed in blood samples collected over 24 h period during different months of the year clearly indicated that maximal values were observed in August, September and October. These findings are in conformity with earlier reports (Plant *et al.*, 1974; Michael *et al.*, 1975; Gordon *et al.*, 1976; Michael and Bonsal, 1977; Beck *et al.*, 1979; Wickings and Nieschlag, 1980) of a circannual rhythmicity in circulating levels of testosterone.

The number of animals responding to electro-ejaculation varied between the months. The least response (4 out of 9) was observed in April and May. A previous study by Wickings and Nieschlag (1980) revealed that none of their animals responded to electro-ejaculation during the summer months. The poor response during the summer months has been related to the drop in circulating androgen levels during this period (Michael and Wilson, 1974). The volume of the seminal fluid collected by electro-ejaculation was also reduced during the summer months which may be indicative of a deprivation of androgenic support to the accessory reproductive organs.
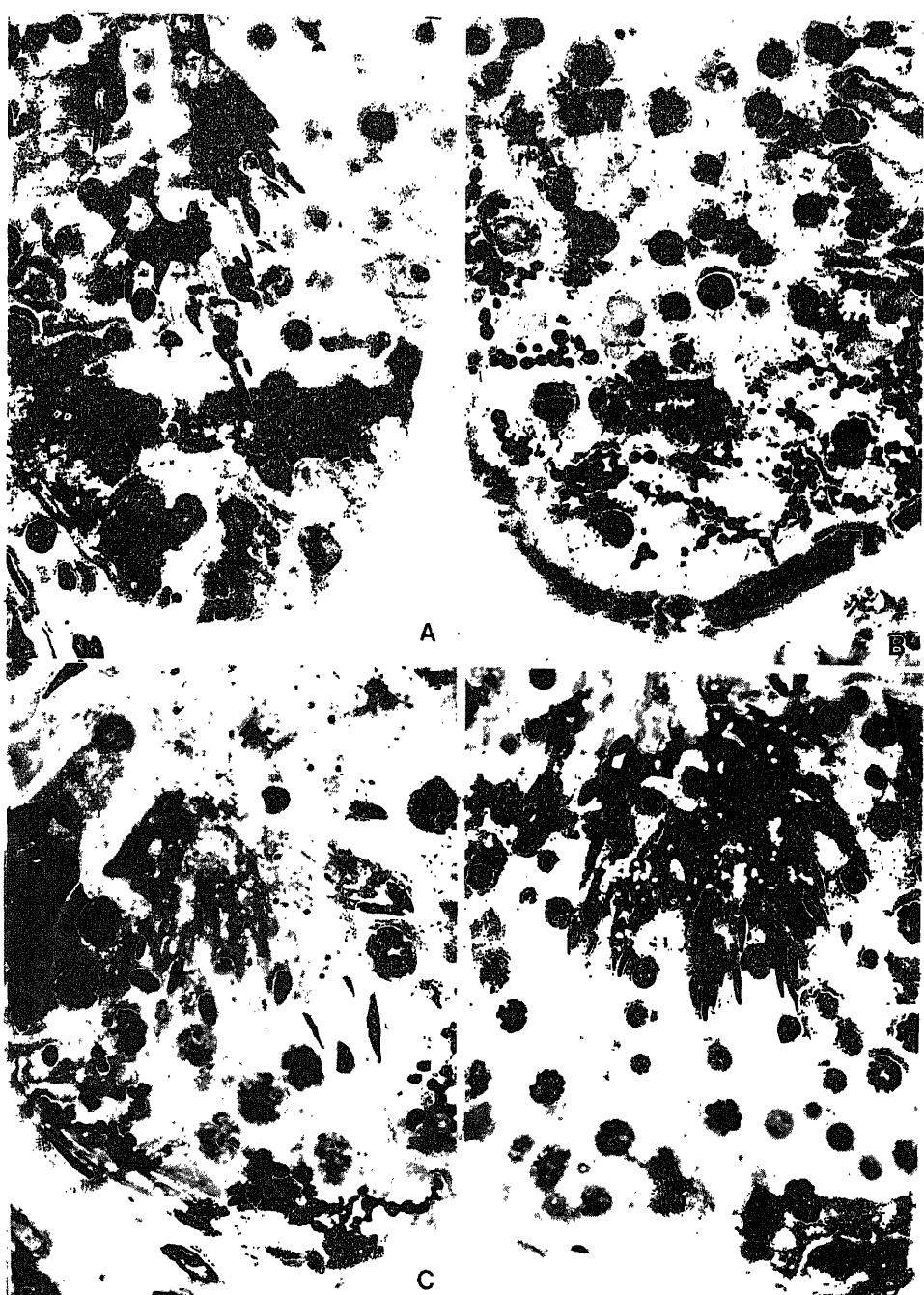
Figure 5. Photomicrographs of araldite sections of testicular biopsies obtained in January (A), April (B), July (C) and October (D) and stained with toludine blue. These photomicrographs illustrate the cyclical changes in the Sertoli cell contents of lipid droplets in relation to the spermatogenic activity. The cells are laden with the lipid droplets when spermatogenesis is quiescent (*e.g.* B and C) and vacuolated during active spermatogenesis (*e.g.* A and D) (×1200).

Spermatogenesis was quiescent from April to August. In conformity with the reduction in spermatogenic activity, the total number of spermatozoa in the electro-ejaculate was markedly also reduced.

Seasonal changes in spermatogenesis have been reported previously in free ranging rhesus monkeys (Conaway and Sade, 1965). The present studies confirm the occurrence of a similar situation in a caged colony of monkeys. Controlled breeding of rhesus monkeys in our colony has revealed that the maximal number of conceptions occurs during the months of September to March (Anand Kumar *et al.*, 1980) which is coincidental with the period when the volume of ejaculated semen and the total number of spermatozoa are high.

The present studies have, for the first time, revealed a seasonal cyclicity in the contents of Sertoli cells. The vacuolation of the Sertoli cells during the period of heightened spermatogenic activity could be related to the nursing role played by the Sertoli cells to the spermatids during spermatogenesis.

The data accrued from the present studies clearly suggest that the evaluation of contraceptive drugs on testicular function of rhesus monkeys needs to be carried out between September and March when the testis is fully functional. Studies carried out during the other months would not be meaningful as the testes would be quiescent. Our data also provide quantitative information on circulating levels of testosterone throughout the year which could be used as a reference background while interpreting the drug-induction effects to be evaluated.

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Effect of oral exposure of *Mycobacterium avium intracellulare* on the protective immunity induced by BCG

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Abstract. The relative protective efficacy of oral administration of mycobacteria as compared to the conventional intradermal route of vaccination has been assessed in guinea pigs. Skin test reactivity to partially purified protein derivative and protective immunity to challenge with virulent *Mycobacterium tuberculosis* were used as parameters of protective immunity.

Oral immunisation of guinea pigs either with BCG or with *Mycobacterium avium intracellulare* induces skin test reactivity and protective immunity comparable to that induced by intradermal route of vaccination. Oral exposure of *Mycobacterium avium intracellulare* prior to oral or intradermal dose of BCG did not interfere with the protective immunity induced by BCG in guinea pigs challenged with *Mycobacterium tuberculosis* $H_{37}Rv$.

Keywords. Protective immunity; BCG; oral immunisation, *Mycobacterium tuberculosis* $H_{37}Rv$; *Mycobacterium avium intracellulare*.

Introduction

The immune response is modulated by the nature, quantity and duration of the immunogen challenge. The oral route is one path by which the immune system comes into contact with an invading organism or its antigens. With recent advances in the immunological concepts there is a growing interest in the effects of oral exposure to antigens (Kagnoff, 1978).

It has long been held that oral intake of mycobacterial antigen can be a means of conferring protective immunity against tuberculosis. Indeed as early as in 1957 the oral administration of BCG to man was shown to confer delayed type hypersensitivity (DTH) (De Assis, 1957). The justification for oral BCG administration was the fact that infection by tubercle bacilli commonly took place by ingestion. Also, it was known that the incompletely differentiated intestinal mucous membrane, present immediately after birth, was permeable to microbial transit towards the lymphatic and blood circulation. Further, autopsy studies proved that BCG passed through the intestinal membrane (Sol Roy Rosenthal, 1980). However, the value of

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oral BCG vaccination in protection against disease was never adequately established in animals or in man, having been superseded by intradermal vaccinaton which can yield high protection (leading article in Tubercle, 1960). Nevertheless, with the failure of intradermal BCG to confer more than a slight protection in some recent clinical trials there is renewed interest in oral BCG (Tuberculosis Prevention Trial, 1980). This interest arises from two different points of view: (i) either oral BCG might be superior to intradermal BCG in conferring protective immunity since it mimicks the natural route of infection and (ii) in some regions of the world, natural oral exposure to environmental mycobacteria might play the role of natural vaccination or even deviate the immune responses thus preventing the protective effect of BCG vaccination.

The latter possibility that prior infection with various non tuberculous mycobateria (NTM) might in some way cross protect against subsequent infection by *Mycobacterium tuberculosis* was examined by Youmans *et al.* (1961). Palmer and Long (1966) tested this hypothesis and found that infection with NTM could provide as much as 50% protective effect as that produced by BCG.

Rook *et al.* (1981) postulated that two types of cell mediated responses could result in persons exposed to certain NTM prior to BCG vaccination; 'the Koch type' and the 'Listeria type' reactions. The former results in blocking the generation of acquired immunity to BCG vaccine whereas the latter enhances the protection. Orme and Collins (1983), using *M. kansasii* in mice found no interference by *M. kansasii* on BCG vaccination. But they speculated that oral immunisation (exposure) with *M. avium intracellulare* (MAI) complex might induce tolerance which might interfere with the immune response to subsequent BCG immunisation.

Among all the atypical mycobacteria isolated and identified so far from the BCG trial area in South India, MAI is the most common (Paramasivan *et al.*, 1985). Hence, in our present study, we have examined whether oral exposure to MAI influences the protective immunity offered by BCG.

Materials and methods

Animals

Random bred male guinea pigs maintained at the Tuberculosis Research Centre and weighing 300–400 g were used.

Experimental design

The experimental schedule is outlined in table 1.

BCG administration

Freeze–dried BCG vaccine, (Central BCG Laboratory, Guindy, Madras) was used for both intradermal and oral vaccination. Two mg of BCG is equivalent to 2.0×10^7 colony forming units.

For intradermal vaccination a dose of 0.075 mg BCG in 0.1 ml was injected intradermally in the shaved right flank. BCG was orally administered with a specially devised L-shaped canula attached to a syringe.

Table 1. Experimental design.

Day '0'	3rd Week	24, 48 and 72 h after skin test	9th Week	15th and 17th Week
Vaccination	Skin test with PPD (3 days before) challenge with H$_{37}$Rv	Skin test reading	Sacrifice, scoring and VC culture	Count colonies

Skin test reactivity to partially purified protein derivative

Ten μg of partially purified protein derivative (PPD) (Weybridge Laboratories, England) in 0·1 ml was injected intradermally on the left flank 3 days before challenge infection. The skin test reaction was assessed after 24, 48 and 72 h and the diameter of the area of induration measured in millimetres.

MAI

One of the MAI strains isolated from the BCG trial area in South India during 1981 was used.

M. tuberculosis strain H$_{37}$Rv

The H$_{37}$Rv strain used was the strain maintained in Tuberculosis Research Centre, Madras. It has been periodically passaged in guinea pigs and is subcultured on Lowenstein Jensen medium (LJ medium).

Assessment of protection after vaccination

Challenge with M. tuberculosis H$_{37}$Rv: All groups of animals were challenged with virulent tubercle bacilli 3 weeks after vaccination, by injecting subcutaneously 1 mg moist weight of the bacilli in 0.5 ml of distilled water in the right thigh of each animal.

Assessment of tubercular lesion: All surviving animals were sacrificed after 42 days and the extent of disease present in the various organs scored after randomisation of the animals. Any animal dying earlier was examined by autopsy and the extent of disease scored. The scoring system of Mitchison *et al.* (1960) was adopted. Morphological scores based on the severity of the disease (No. of tubercles, areas of necrosis and caseation) for the spleen were 0, 10, 20, 30 and 40; for the liver: 0, 8, 15, 23 and 30; for the lungs: 0, 5, 10, 15 and 20; and, for the site of inoculation and its draining lymphnodes, values were from 0–10 depending on the extent of involvement of the regional glands. Thus, macroscopic scores ranged from a minimum of 0 to a maximum (of 100) score for all organs of severely diseased animals of 100.

The mean root index: The mean root index (MRI) was obtained by dividing the total score by the number of days of survival of the animals (irrespective of whether

they died or were killed) and taking its square root. The root index of disease thus served as a measure of the degree of protection afforded by vaccination, the smaller the root index, the greater the protection.

Colony counts on spleen homogenates: A portion of the spleen taken aseptically from the animal was weighed and homogenised in 5 ml distilled water. The number of colony forming units (cfu) was determined by inoculating LJ medium with serial dilutions of the homogenate. Colonies were counted 6 and 8 weeks after incubation. The viable count (VC) was calculated as follows:

$$VC = cfu \times \frac{\text{Total wt. of spleen}}{\text{Wt. of portion taken}} \times \frac{1}{\text{Vol. inoculated-LJ medium}} \times 5.$$

Results

Table 2 shows the result of various doses of oral BCG administration on DTH and protection against challenge with *M. tuberculosis* H[37]Rv. The intradermally vaccinated animals developed skin test reactivity (mean induration of 12.7 mm) and showed a MRI of 0.78. The mean log viable count of bacilli in the spleen was 4.3. None of the animals receiving oral doses ranging from 0·075–6 mg developed skin test reactivity or protective immunity.

Table 2. Lack of effect of smaller doses of oral BCG immunisation on DTH and protection against challenge with H_{37}Rv.

Group[a]	Skin test induration in mm[b] Mean ± S.D.	MRI[c] ± S.D	Log VC spleen Mean ± S.D.
Control	0	1·26 ± 0·13	6·3 ± 0·45
0·075 mg (ID)	12·7 ± 4·0	0·78 ± 0·19	4·3 ± 0·60
0·075 mg (oral)	0	1·17 ± 0·12	6·0 ± 0·53
1·5 mg (oral)	0	1·19 ± 0·12	6·7 ± 0·43
3·0 mg (oral)	0	1·21 ± 0·12	6·1 ± 0·42
6·0 mg (oral)	0	1·11 ± 0·43	5·6 ± 0.15

[a] 6 animals per group.
[b] 10 μg PPD was injected intradermally and readings were taken at 24, 48 and 72 h.
[c] Mean of the square root of total score divided by No. of survival days.

Since a single oral dose of 6 mg of BCG failed to sensitize or confer immunity the effect of a large single dose (30 mg) or 6 multiple doses (consecutive days) of 5 or 10 mg were tested and the results are summarised in table 3. Both groups of animals developed skin test reactivity and protective immunity.

Subsequently, the effect of oral immunisation with MAI was studied using a single dose of 30 mg. Table 4 shows that, the guinea pigs which were given either intradermal or oral BCG immunisation showed a positive skin test reaction and a lower MRI. Similar results were seen with animals exposed to oral MAI.

The influence of MAI on BCG induced protective immunity when given orally prior to BCG is shown in table 5. The animals which were exposed to MAI prior to

Table 3. DTH responsiveness and protection against challenge with *M. tuberculosis* H_{37}Rv after larger doses of oral BCG.

Group[a]	Skin test induration (mm)	MRI ± S.D.	Log VC spleen Mean ± S.D.
Experiment No. 1			
Control	1·0 ± 1·0	1.0 ± 0·03	5·74 ± 0·4
BCG			
0·07 mg (ID)	18·6 ± 2·0	0·71 ± 0·10	4·70 ± 0·4
5·0 mg '0' × 6 days	15·0 ± 0	0·65 ± 0·16	4·9 ± 0·5
10·0 mg '0' × 6 days	16·0 ± 2·0	0·74 ± 0·15	4·6 ± 0·9
30 mg oral Single dose	14·0 ± 2·0	0·76 ± 0·03	4·77 ± 0·4
Experiment No. 2			
Control	1·5 ± 1·0	1·42 ± 0·17	6·00 ± 0·3
BCG			
0·075 (ID)	13·7 ± 2·0	0·66 ± 0·23	4·90 ± 0·2
30 mg (oral)	11·3 ± 2·0	0·68 ± 0·10	4·40 ± 0·31

[a] 6 animals in each group.

Table 4. DTH and protective immunity induced by MAI.

Group[a]	Skin test Mean ± S.D. (mm)	MRI ± S.D. (mm)
Control	2·4 ± 1·5	1·4 ± 0·17
BCG (intradermal)	9·2 ± 2·2	0·96 ± 0·095
BCG (oral/30 mg)	10 ± 1·6	0·98 ± 0·01
MAI (oral/30 mg)	7·8 ± 1·9	0·72 ± 0·29

[a] 5 animals in each group.

BCG developed skin test reactivity (induration of 17.3 mm) and showed a MRI of 0·52.

Table 6 shows the influence of MAI on BCG induced protective immunity when given orally 3 weeks prior to intradermal BCG. The control animals gave a MRI of 0·95 and a viability count of 17×10^3. In contrast, the group of animals which received BCG (orally alone) and the group which received MAI (orally) prior to intradermal BCG showed a lower MRI and lower viability count when compared to those of controls ($P < 0·05$).

Table 5. Influence of MAI on BCG induced protective immunity when given orally 3 weeks prior to oral BCG.

Group[a]	Skin test Mean ± S.D. (mm)	MRI ± S.D. (mm)
Control	1·3 ± 2·3	0·86 ± 0·07
BCG (oral/30 mg)	17·6 ± 0·57	0·50 ± 0·27
M. avium (oral/30 mg)	13·6 ± 2·3	0·47 ± 0·07
M. avium (3 weeks prior to BCG immunisation)	17·3 ± 2·0	0·52 ± 0·09

[a] 3 animals in each group.
P values—Control *versus* BCG (oral) < 0·05;
Control *versus* *M. avium* < 0·05;
Control *versus* *M. avium*
3 weeks prior to BCG < 0·05.

Table 6. Influence of prior oral exposure of MAI on BCG induced protective immunity.

Group[a]	MRI ± S. D.[b]	Viable count[c]
Control	0·95 ± 0·12	$17 \times 10^3 \pm 5·8 \times 10^3$
BCG (oral/30 mg)	0·65 ± 0·15	$7·6 \times 10^3 \pm 3·6 \times 10^3$
Oral MAI (3 weeks prior to ID BCG)	0·61 ± 0·2	$3·9 \times 10^3 \pm 1·78 \times 10^3$

[a] Consists of 5 animals in each group.
[b] *P* value is significant when MRI of oral BCG immunised animals and the animals which received oral MAI and intradermal BCG were compared with control group(< 0·05).
[c] *P* value is significant when viability count of oral BCG immunised animals and the animals which received oral MAI and intradermal BCG were compared with control group (< 0·05).
When the MRI and the VC of oral BCG immunised animals were compared with those of the animals which received oral MAI and subsequent intradermal BCG there was no significant difference.

Discussion

High prevalence of atypical mycobacterial infection with resultant sensitization in the population of the BCG study area in Chingleput District, has been implicated as one of the mechanisms for the lack of protection by BCG against adult type of tuberculosis. The present study was carried out to examine the hypothesis, that oral exposure to MAI influences the protective immunity offered by BCG.

The initial experiments were set up to establish the protective immunity offered by oral BCG. Three weeks after oral immunisation with BCG, guinea pigs

developed marked skin test reactivity to PPD-S. A single oral dose of live BCG upto 6 mg weight did not induce skin test positivity whereas in separate experiments a dose of 30 mg was effective.

As there is no immunological test, either *in vivo* or *in vitro*, that correlates fully with protective immunity, an *in vivo* challenge with virulent *M. tuberculosis* H_{37} Rv was chosen as the means to examine protective immunity. It was seen that doses of BCG that did not confer delayed type hypersensitivity did not confer protection and protection was always seen when DTH was present. Nevertheless, there was no correlation between extent of delayed type hypersensitivity and degree of immunity as revealed by either lower viability count or lower disease indices.

Oral immunisation with MAI resulted in a mean skin induration of 7.8 mm as compared to 10 mm and 9.2 mm in animals given BCG orally or intradermally. This difference in skin test induration to PPD-S bears no relationship to MRI because MAI infected animals gave the lowest MRI as compared to those in animals given oral or intradermal BCG. Similar observations were obtained in groups of animals primed with MAI prior to BCG administration.

Rook *et al* (1981) proposed the hypothesis that contact with NTM might in some way jeopardise or interfere with the generation of acquired cell mediated immunity resulting from BCG immunisation and hence provide an explanation for the failure of the recent BCG trial in South India. Orme and Collins, (1983) concluded from their experiment that despite the presence of *M. kansasii* infection BCG vaccinated animals were fully resistant to subsequent aerosol challenge with virulent *M. tuberculosis*. Continuing in a similar line Orme and Collins (1984), using animals with pulmonary infection by *M. kansasii*, *M. Simiae*, *M. avium* and *M. scrofulaceum* showed that subsequent intravenous inoculation with 10^6 BCG had no discernible effect on the course of NTM infection within the lungs. However, all the BCG vaccinated group were fully resistant to subsequent acute aerogenic challenge with *M. tuberculosis* regardless of the presence of pulmonary NTM infection. Edwards *et al*. (1982) and more recently Smith *et al* (1985) reported that there was no interference due to MAI on the protective efficacy of BCG by using an aerosol challenge model in the guinea pigs. Collins (1983) nevertheless hypothesised that oral infection caused by MAI might induce immunological perturbation within the host capable of interfering with subsequent immune response.

Results of the present study using guinea pigs as model system, showed that oral exposure with MAI did not interfere with the protective immunity induced by BCG.

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Diagnostic utility of monoclonal antibodies raised against microfilarial excretory-secretory antigens in bancroftian filariasis

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Abstract. Two monoclonal antibodies *Wuchereria bancrofti* E 33 and *Wuchereria bancrofti* E 34 raised against *Wuchereria bancrofti* microfilarial excretory-secretory antigens were studied for their diagnostic utility. *Wuchereria bancrofti* E 34 monoclonal antibody was found to be relatively specific and sensitive in detection of circulating filarial antigen. When *Wuchereria bancrofti* E 34 monoclonal antibody was used alongwith immunoglobulin G fraction of human filarial serum immunoglobulins in double antibody sandwich enzyme linked immunosorbent assay, 68% of microfilaraemic sera (26 out of 38), 12% of clinical filarial sera (3 out of 25), 13% endemic normal sera (2 out of 15) and none of the 20 non-endemic normal sera showed the presence of filarial antigen. The filarial antigen detected by *Wuchereria bancrofti* E 34 monoclonal antibody in double antibody sandwich enzyme linked immunosorbent assay is possibly associated with the active stage (microfilaraemia) of infection.

Keywords. *Wuchereria bancrofti*; bancroftian filariasis; excretory-secretory antigens; monoclonal antibodies; enzyme linked immunosorbent assay; circulating filarial antigen

Introduction

In immunoparasitology, recent studies have attempted to develop diagnostic methods based on the detection of parasite antigen. Compared to conventional methods based on antibody detection, these tests are more informative in detecting the active infection and in assessment of effectiveness of chemotherapy. Filarial antigen has been demonstrated in the sera and urine samples of infected humans and animals (Dasgupta and Shukal Bala, 1978; Desowitz and Una, 1976; Dissanayake *et al.*, 1982; Hamilton *et al.*, 1984; Kaliraj *et al.*, 1979; Reddy *et al.*, 1984a) using antisera raised against heterologous and homologous filarial antigens. The utility of immunoglobulin fraction of clinical filarial serum was explored for the detection of circulating filarial antigen (Kaliraj *et al.*, 1981; Reddy *et al*, 1984b). Hybridoma derived monoclonal antibody is another reagent with great potential

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for detection of specific antigen of interest. Dissanayake *et al.* (1984) and Forsyth *et al.* (1985) have explored the utility of one of the monoclonal antibodies (*Gib* 13) raised against *Onchocerca gibsoni* in detection of filarial antigen in sera and urine samples using immunoradiometric assay (IRMA). This communication reports the detection of circulating antigen in sera of filarial patients employing the monoclonalantibodies raised against *Wuchereria bancrofti* microfilarial excretory-secretory antigens (*Wb* mf ES Ag).

Materials and methods

Sera

Human sera (98 samples) belonging to different groups *viz.*, normal subjects from endemic and non-endemic regions and filarial (microfilaraemia and clinical filariasis) were screened in this study. Filarial blood samples were collected from Sevagram and surrounding villages which are endemic for nocturnally periodic form of *W. bancrofti*. Endemic normal samples were from healthy individuals living in Sevagram and its surrounding villages and having neither microfileraemia (mf) in their blood nor any clinical symptoms. The presence or absence of mf was confirmed by night blood (wet smear) examination. Non-endemic normal blood samples were collected from students of this Institute coming from places like Chandigarh, Kashmir etc. where there is no filariasis. Sera were separated from blood samples and stored at −20°C after addition of sodium azide as preservative.

Wb mf ES Ag

Wb mf Es Ag was prepared as described by Kharat *et al.* (1982) and concentrated 200 fold by ultrafiltration and freezedrying.

Preparation of monoclonal antibodies against Wb mf ES Ag

Monoclonal antibodies were raised against *Wb* mf ES Ag as described earlier (Reddy *et al.*, 1984c). The monoclonal antibodies *Wb* E 33 and *Wb* E 34 which were relatively more reactive with *Wb* mf ES Ag were selected for evaluating their diagnostic utility in filariasis. The monoclonal antibodies were concentrated from culture supernatants by 50% saturation with ammonium sulphate. The conjugation of concentrated monoclonal antibody (20 mg) and 1000 units of enzyme penicillinase (Sigma Chemical Co., USA) was achieved by the single step method of Avrameas (1969) using glutaraldehyde.

Immunoglobulin G fraction of human filarial serum immunoglobulins

Immunoglobulin G fraction of human filarial serum immunoglobulins (FSIgG) was prepared from pooled clinical filarial sera showing anti-microfilarial soluble antigen antibody by ammonium sulphate precipitation followed by DEAE-cellulose (Whatman DE 52) chromatography as described by Reddy *et al.* (1984b). Twenty mg of FSIgG was conjugated to 1000 units of enzyme penicillinase (Sigma Chemical Co., USA) by the method of Avrameas (1969).

Sandwich enzyme linked immunosorbent assay

Sandwich enzyme linked immunosorbent assay (ELISA) was carried out as described by Reddy *et al.* (1984b). The optimal dilutions of all the reagents were determined by chequer board titration. The wells of the polyvinylchloride (PVC) microtitre plates (Dynatech Laboratory Inc., Singapore) were sensitized with optimal concentration of monoclonal antibody (10 μg/ml) in carbonate buffer (0·06 M, pH 9·6) at 37°C for 3 h and then drained. The plate was further incubated with 200 μl of 3% (w/v) bovine serum albumin (BSA) in carbonate buffer at 37°C for 2 h. After washing the plate 5 times with phosphate buffer saline (0·01 M, pH 7·2) containing 0·05% (v/v) tween 20 (PBS/T), 100 μl of optimally diluted test sera (1:300) in PBS/T were added and incubated at 37°C for 3 h. After washing the plate again, 100 μl of penicillinase conjugate of the same monoclonal antibody (used for sensitizing the plate) was added and incubated at 37°C for 3 h. Following the final washing, the immune reaction was observed by incubating the wells with 100 μl of substrate solution at 37°C for 30 min. The substrate consisted of soluble starch (150 mg) in 27·5 ml of 0·25 M sodium phosphate buffer (pH 7·2) containing 10·6 mg penicillin V, and 65 μl of 0·08 M iodine in 3·2 M potassium iodide solution. The substrate was prepared fresh before use.

The enzyme reaction was terminated by adding 25 μl of 5 N HCl, and the results were evaluated visually. The complete decolorization or decolorization with a slight tinge of substrate colour denoted a positive reaction, while negative reaction was confirmed by persistance of blue colour.

Similarly sandwich ELISA was also done using FSIgG and FSIgG penicillinase combination.

Double antibody sandwich ELISA

Double antibody sandwich ELISA using FSIgG and *Wb* E 34 monoclonal antibody penicillinase conjugates was done with modification in incubation steps as described for two site IRMA by Dissanayake *et al.* (1984) and Forsyth *et al.* (1985).

The wells in the PVC microtitre plate were incubated with 100 μl of optimal concentration of FSIgG (25 μg/ml) in carbonate buffer (0·06 M, pH 9·6) at 4°C for overnight and then drained. The wells were further incubated with 200 μl of 3% BSA in carbonate buffer at 37°C for 2 h, followed by washing with PBS/T. While the BSA incubation was going on, optimally diluted test sera (1:150) in PBS/T were pre-incubated with equal volumes of optimally diluted *Wb* E 34 monoclonal antibody penicillinase conjugate (1:200) in PBS/T in small plastic vials at 37°C for 3 h. The reaction mixture (100 μl) was then transferred to FSIgG coated wells and incubated at 37°C for 3 h followed by washing with PBS/T. The immune reaction was then observed by incubating with 100 μl of substrate and the results were evaluated as described above for sandwich ELISA.

Results

A total number of 64 sera belonging to different groups were screened in sandwich ELISA using the same monoclonal antibody on either side and the results are summarized in table 1. When *Wb* E 34 monoclonal antibody was used, none of the

Table 1. Detection of circulating filarial antigen using the same monoclonal antibody on both the sides in sandwich ELISA.

Sera	No. exam.	No. showing positive reaction* using the monoclonal antibody	
		Wb E 33	*Wb* E 34
Nonendemic normal (mf − ve)	12	3	0
Endemic normal (mf − ve)	20	2	2
Microfilaraemia (mf + ve)	20	10	8
Clinical filariasis (mf − ve)	12	4	2

* Sera showing positive reaction for filarial antigen at a dilution of 1:300.

12 nonendemic normal sera, only 2 out of 20 endemic normal sera (10%), 8 out of 20 microfilaraemic sera (40%) and 2 out of 12 clinical filarial sera (17%) were positive for filarial antigen. Though the *Wb* E 33 monoclonal antibody detected filarial antigen in higher number of microfilaraemic (10 out of 20) and clinical filarial sera (4 out of 12), it showed nonspecific reactions with 3 out of 12 nonendemic normal sera. *Wb* E 34 monoclonal antibody was used together with FSIgG in double antibody sandwich ELISA and a total of 98 sera were analysed (table 2). Filarial antigen was detected in none of the 20 nonendemic normal sera, 2 out of 15 endemic normal sera (13%), 26 out of 38 microfilaraemic sera (68%) and 3 out of 25 clinical filarial sera (12%). The same 98 sera were also analysed in

Table 2. Detection of circulating filarial antigen in double antibody sandwich ELISA and FSIgG sandwich ELISA.

Sera	No. exam.	Sera showing positive reaction*			
		Double antibody sandwich ELISA using FSIgG and *Wb* E 34 monoclonal antibody		FSIgG sandwich ELISA	
		No.	Per cent	No.	Per cent
Nonendemic normal (mf − ve)	20	0	0	0	0
Endemic normal (mf − ve)	15	2	13	3	20
Microfilaraemia (mf + ve)	38	26	68	28	74
Clinical filariasis (mf − ve)	25	3	12	13	52

* Sera showing positive reaction for filarial antigen at a dilution of 1:150 in double antibody sandwich ELISA and 1:300 in FSIgG sandwich ELISA.

sandwich ELISA using FSIgG and FSIgG penicillinase and the results are compared with double antibody sandwich ELISA (table 2). In both the assay systems, none of the 20 non-endemic normal sera were positive for filarial antigen. While the percentage positivity in microfilaraemic and endemic normal sera was more or less same in both the assay systems, only 12% clinical filarial sera were positive in double antibody sandwich ELISA, compared to 52% positivity in FSIgG sandwich ELISA.

Discussion

Out of the 2 monoclonal antibodies (*Wb* E 33 and *Wb* E 34) which were more reactive to *Wb* mf ES Ag, when evaluated in sandwich ELISA using the same monoclonal antibody on either side, *Wb* E 34 was found to be relatively specific than *Wb* E 33 and showed no cross reactions with any of the 20 nonendemic normal sera screened. However, using *Wb* E 34 monoclonal antibody, the sensitivity was found to be limited as only 40% microfilaraemic sera and 17% clinical filarial sera were positive for filarial antigen. Earlier studies from our laboratory (Kaliraj *et al.*, 1981; Reddy *et al.*, 1984b) have shown the FSIgG, a polyclonal antibody isolated from clinical filarial serum to be useful in detection of circulating filarial antigen. Hence *Wb* E 34 monoclonal antibody was used together with FSIgG in double antibody sandwich ELISA. With the inclusion of FSIgG, the number of microfilaraemic sera positive for filarial antigen was considerably increased from 40% in sandwich ELISA to 68% in double antibody sandwich ELISA. However, still in about one third of microfilaraemic sera, the circulating filarial antigen could not be detected, as is the case in most of the antigen assays reported in filariasis (Ouaissi *et al.*, 1981; Desmoutis *et al.*, 1983; Reddy *et al.*, 1984b). This can be explained by the expected interference of the excess of antibodies which keep the antigen in immunecomplex form saturating the antigen sites and possibly leading to its clearance from circulation. Filarial antigen was detected in 13% of endemic normal persons in this assay system. These people may be having prepatent or asymptomatic infection. In contrast, about 50% of this group showed filarial antigen when *Gib* 13 monoclonal antibody raised against heterologous filarial antigen (*O. gibsoni*) was used by Dissanayake *et al.* (1984) and Forsyth *et al.* (1985).

The results of FSIgG sandwich ELISA (table 2) agree with our previous observations using the same assay system (Reddy *et al.*, 1984b). However, compared to the results of FSIgG sandwich ELISA, the higher percentage of positivity in microfilaraemic sera (68%) than in clinical filarial sera (12%) in double antibody sandwich ELISA suggests that the antigen recognized by *Wb* E 34 monoclonal antibody is possibly associated with the active stage of infection. Thus the double antibody sandwich ELISA, using the polyclonal antibody FSIgG and *Wb* mf ES Ag monoclonal antibody *Wb* E 34, may be useful in detecting the circulating antigen in active infection.

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Biochemical changes in pigeonpea (*Cajanus cajan* (L.) Millsp.) leaves in relation to resistance against sterility mosaic disease*

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Abstract. Changes in different biochemical parameters like total phenolic content, protein pattern, polyphenol oxidase, peroxidase and isozymes of peroxidase were compared in sterility mosaic resistant (Hy3C) and susceptible (Type-21) pigeonpea varieties at different growth stages both under inoculated and uninoculated conditions. Resistant variety was characterized by the presence of specific isoperoxidase and proteins but only little difference was recorded between resistant and susceptible variety with respect to preformed or induced total phenolics and peroxidase activity. The activity of polyphenol oxidase increased substantially in susceptible variety following infection. Role of these changes is discussed in relation to disease resistance.

Keywords. Pigeonpea; pigeonpea sterility mosaic virus; phenolics; peroxidase; polyphenol oxidase.

Introduction

Sterility mosaic of pigeonpea, considered to be viral in etiology (Nene, 1980), is one of the most serious diseases of the crop. The pigeonpea sterility mosaic virus (PSMV) is transmitted and perpetuated through a mite, *Aceria cajani* (Nene and Rathi, 1972).

Certain resistant germplasm lines have been made available to the pulse breeders in the recent past (Nene and Reddy, 1976) and no information is available on the biochemistry of resistance mechanism in these germplasms. A role for phenolics and phenol oxidizing enzymes like polyphenol oxidases (PPO) and peroxidases in plant resistance against viral diseases have been implicated by several workers, but also contradicted by others (Loebenstein, 1972; Fric, 1976). Therefore, the present investigation was undertaken to find out the levels of total phenolics and their oxidizing enzymes in the leaves of two lines of pigeonpea in relation to their resistance against PSMV.

Materials and methods

Plant material

Two varieties of pigeonpea (*Cajanus cajan* (L.) Millsp.), one highly susceptible (Type-21) and other resistant (Hy3C) to PSMV, were obtained from International Crop Research Institute for Semi-Arid and Tropics (ICRISAT), Patancheru (Hyderabad). They were raised in glass house in earthen pots filled with a mixture of soil and farm yard manure (10:1). Fifteen surface sterilized seeds were planted in each pot (30 cm) and after emergence, 10 seedlings per pot were maintained.

Inoculation

The inoculum of PSMV was obtained from naturally infected plants of pigeonpea cv. Sharda. The diseased leaflets infested with the mites served as inoculum. The seedlings were inoculated at the two leaf stage (*i.e.* 12 day old) by following the 'leaf pinning technique'. Two leaflets from diseased plant having sufficient number (20–50 mites per leaflet) of eriophyid mites were pinned with the help of Entomological pin No 20 (F. F. Taylor and Co., Birmingham, UK) to each primary leaf of seedling in such a manner that the healthy leaf was sandwitched between the lower surfaces of the two infected leaflets. Control plants were pinned in the same manner with healthy leaflets. In order to obtain better infection, inoculated plants were kept in shade for 12 h to facilitate the migration of more mites to healthy leaves. After 5 days of inoculation, the leaflets pinned to the healthy leaf were removed and the entire plant was sprayed with 0·1% metasystox.

Sampling

The primary leaves and first trifoliate of same size and position were harvested after washing them with distilled water from both inoculated and control plants after 0, 7, 14 and 28 days of inoculation. The harvested leaves were immediately transferred to ice bags and pooled replication wise. Three replications were taken for each interval. A part of each lot was used for the electrophoretic separation of soluble proteins and peroxidase (PO) isozymes. Half of the rest was weighed and stored at $-20°C$ to determine the PO and PPO activities. The remaining half of the each lot was oven dried at 60°C for 48 h, powdered with the help of mortar and pestle and stored in desiccator for the quantitative estimation of phenolic compounds and soluble proteins.

Determination of total phenolic content

Phenolic compounds were extracted from leaves using the method as described by Kuc *et al.* (1956). AOAC colorimetric method (AOAC, 1965) was followed for the quantitative estimation of total phenolic content. Tannic acid was used as standard.

Determination of PO activity

Peroxidase was extracted and assayed by the method of Retig (1974). Guaiacol was used as substrate. One unit is defined as the amount of enzyme that caused an increase in $A470$ of 0·01 in 1 min at 30°C.

Determination of PPO activity

PPO was extracted and assayed by the method of Tripathi *et al.* (1975) using pyrocatechol as a substrate. One unit of enzyme is defined as the amount of enzyme that caused an increase in A410 of 0·01 in 1 min at 30°C.

Electrophoretic separation of soluble proteins and PO isozymes

The first leaves were crushed in chilled mortar and pestle with 0·2 M cold phosphate buffer (pH 7·0). The homogenate was centrifuged at 14000 *g* for 40 min at 4°C. The soluble proteins and PO isozymes were separated on 7·5% polyacrylamide gel and subsequently stained by following the method of Davis (1964) except that ammonium persulphate was used as a polymerizing agent in the spacer gel instead of riboflavin. About 0·1 mg protein was loaded in each gel. Proteins were stained in Coomassie blue while peroxidase isozymes were detected by staining with benzidine and H_2O_2.

Results

Total phenols

As shown in table 1, total phenolic content of healthy leaves of resistant variety was significantly·higher than that of the susceptible one at all the stages of sampling except at 21 and 28 days where differences were non-significant. Phenolic content of the leaves increased progressively with increasing plant age upto 21 days after inoculation, thereafter, it declined in both the varieties.

Upon inoculation, phenolic content increased in both resistant and susceptible varieties. However, the magnitude of increase was higher in the resistant variety than in the susceptible one particularly during initial intervals *i.e.* 7 and 14 days after inoculation (table 1).

Protein pattern

A total of 34 different protein bands were observed at different stages of sampling but none of the two varieties showed all 34 bands at any stage (figure 1). Proteins appearing at band positions: 1, 2, 6, 8, 10, 13, 14, 18, 19, 21, 23 and 31 were found in both the varieties at one stage or other. A group of 6 proteins was Hy3C specific, appearing at band positions: 5, 12, 16, 24, 27 and 33. Four proteins, band positions: 11, 26, 30 and 32, were specific to Type-21. Certain protein bands (1 at 14 day and 19 and 21 at 21 day) were stage specific (figure 1).

Peroxidase

Irrespective of the stage of sampling higher PO activity was recorded in healthy leaves of resistant variety than in the susceptible one. However, differences were significant only at 14 and 21 day stages. The activity of enzyme was observed to increase with age of the leaves in both the varieties (table 2) but the increase was slight and non-significant between any two consecutive intervals.

Upon inoculation with PSMV, initially there was a slight increase in the enzyme activity in the leaves of both susceptible and resistant varieties, but after 14 days, it

Table 1. Total phenolic content in the leaves of two pigeon pea varieties inoculated with pigeon pea sterility mosaic virus.

Days after inoculation	Type-21			Hy3C		
	Uninocu- lated	Inocu- lated	Increase (+) or decrease (−) over uninoculated (%)	Uninocu- lated	Inocu- lated	Increase (+) or decrease (−) over uninoculated (%)
0	9·40*	9·00	−4·25	10·50	10·35	−1·61
7	10·05	11·05	+9·95	10·86	12·90	+18·78
14	10·55	11·45	+8·53	11·41	12·65	+10·86
21	11·95	12·10	+1·25	11·81	12·20	+3·30
28	11·00	10·35	+5·90	11·66	11·85	+1·62

* Phenolic compounds (mg per gram of dry leaf).
l.s.d. ($P = 0.05$) = 0.99.
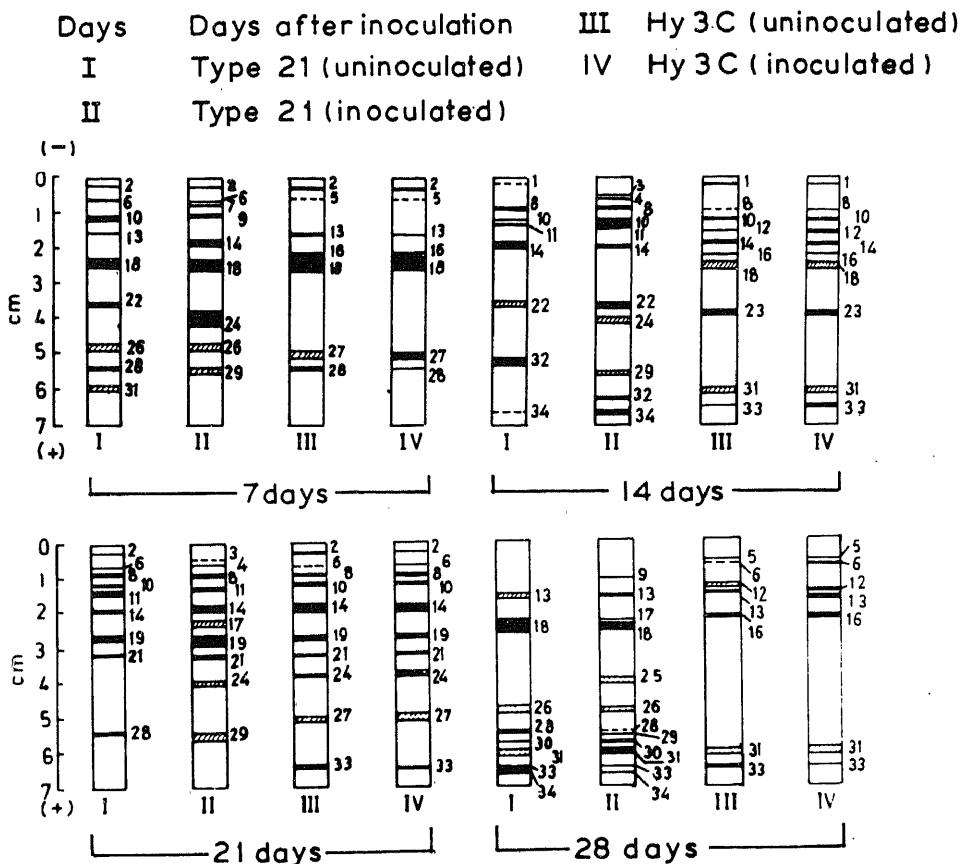
Figure 1. Protein pattern of pigeonpea leaves of susceptible (Type 21) and resistant (Hy3C) varieties at different intervals after inoculation with PSMV.

recorded significant decline in the susceptible variety falling below the control level at the following stages *i.e.* at 21 and 28 days after inoculation. In resistant variety, however, activity continued to increase (statistically not significant) till 21 days after inoculation, followed by a decline thereafter (table 2).

Isozymes of peroxidase

In the healthy leaves of resistant variety 7 (a, b, c, d, e, f and h) and in the susceptible, 6 (a, b, d, e, f and h) isozymes of PO were detected at 7, 14 and 21 day stages. One additional isozyme 'g' was added in susceptible variety at 28 day stage. Bands c and g were specific to resistant and susceptible varieties, respectively. Upon inoculation of PSMV, no quantitative deviation from the control was observed in the isozymes of PO of resistant variety while many such changes were recorded in susceptible one (figure 2). Intensity of all the bands diminished after infection except band 'a' whose width and intensity remained unaltered.

Table 2. Peroxidase activity in the leaves of two varieties of pigeon pea inoculated with pigeon pea sterility mosaic virus

Days after inoculation	Type-21			Hy3C		
	Uninocu-lated	Inocu-lated	Increase (+) or decrease (−) over uninoculated (%)	Uninocu-lated	Inocu-lated	Increase (+) or decrease (−) over uninoculated (%)
0	3869*	3843	−0·7	3993	3994	+1·7
7	3923	4081	+4·0	4091	4166	+4·1
14	4004	4121	+2·9	4198	4324	+3·0
21	4039	3746	−7·2	4293	4404	+2·6
28	4209	3493	−17·0	4274	4349	+0·7

* Peroxidase activity (enzyme units per gram of fresh tissue) in leaves. One unit indicates amount of enzyme required to increase in A470 by 0·01 per min at 30°C.
l.s.d. $(P = 0.05) = 145$.

Table 3. Polyphenoloxidase activity in the leaves of two varieties of pigeon pea inoculated with pigeon pea sterility mosaic virus.

Days after inoculation	Type-21			Hy3C		
	Uninocu-lated	Inocu-lated	Increase (+) or decrease (−) over uninoculated (%)	Uninocu-lated	Inocu-lated	Increase (+) or decrease (−) over uninoculated (%)
0	104*	98	−5·7	111	110	−0·9
7	118	149	+26·0	131	139	+6·2
14	129	203	+56·5	144	148	+3·0
21	115	201	+74·5	124	127	+2·5
28	134	218	+62·2	138	143	+3·9

* Polyphenoloxidase activity (enzyme units per gram of fresh tissue) in leaves. One unit indicates amount of enzyme required to increase in A410 by 0·01 per min at 30°C.
l.s.d. $(P = 0.05) = 13$.
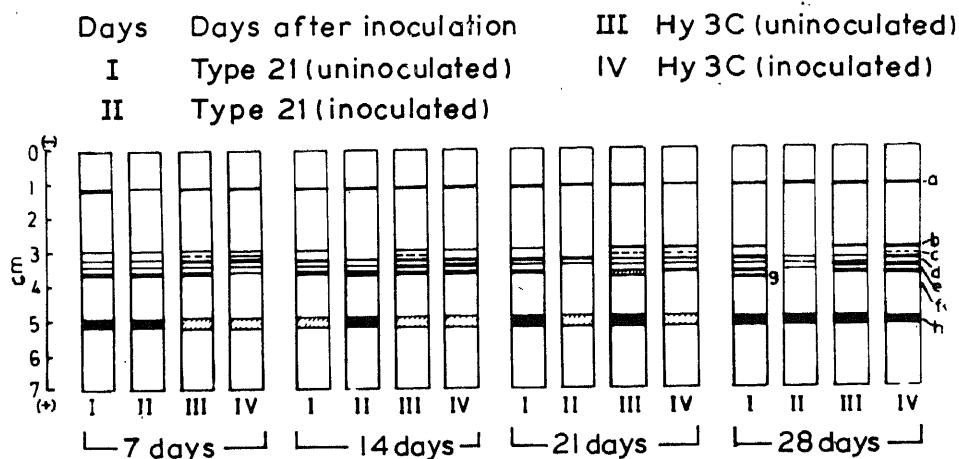
Figure 2. Isozyme pattern of PO in pigeonpea of susceptible (Type 21) and resistant (Hy3C) varieties at different intervals after inoculation with PSMV.

Polyphenol oxidase

Healthy leaves of resistant variety Hy3C, exhibited slightly higher PPO activity than the corresponding leaves of susceptible variety, Type-21, at all the stages of sampling (table 3), but the differences were statistically not significant for most of the intervals except day 14.

Inoculation resulted in significantly increased PPO activity in susceptible variety but not in the resistant one. Unlike healthy leaves, inoculated leaves of susceptible variety exhibited significantly higher PPO activity in comparison with the corresponding leaves of resistant variety at all the stages of sampling except at day 0 (table 3).

Discussion

Constitutive phenolics and their oxidizing enzymes like PO and PPO have been implicated in resistance in certain viral diseases (Fric, 1976; Kosuge, 1969; Loebenstein, 1972; Tripathi *et al.*, 1975). However, difference between resistant and susceptible varieties of pigeonpea with respect to these parameters was too little to be of any significance in providing resistance against PSMV. Similar observations have been recorded in certain other viral diseases (Barbara and Wood, 1972; Cabanne *et al.*, 1971; Fric, 1976).

Upon inoculation with PSMV, the only appreciable change recorded was the activity of PPO in susceptible variety where it increased considerably after 7 days of inoculation and continued to increase till 28 days after inoculation. In the resistant variety its activity was practically unaffected following inoculation. This indicates the possible involvement of PPO in symptom expression as also indicated by few other investigations (Barbara and Wood, 1972; Suseno and Hampton, 1966).

Constitutive isoperoxidases appearing at position 'c' and 'g' were unique for the resistant and susceptible plants, respectively. Altered zymogram pattern of isoperoxidases of susceptible variety, following infection with PSMV, suggested inactivation of existing isoperoxidases, activation of inactive form and/or synthesis of new isoperoxidases. No such alterations were recorded in resistant variety.

The difference in protein pattern of the two genotypes might be due to their genetic differences. Some of the specific proteins may also be associated with susceptibility or resistance to PSMV. Resistance associated proteins are reported in several virus-host systems (Sela, 1981).

The present findings indicate non-involvement of total phenols, PO and PPO in imparting resistance to pigeonpea against sterility mosaic disease. PPO may be involved in or associated with symptom expression. Proteins specific to resistant variety were observed. However, the question whether these proteins are varietal specific or are involved in imparting resistance could not be ascertained.

Acknowledgement

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An indirect haemagglutination test to detect serum antibodies to *Giardia lamblia*

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Abstract. We used an indirect haemagglutination test with *Giardia lamblia* trophozoites as the antigen to detect anti-*Giardia lamblia* antibodies in serum, the soluble tritonated *Giardia lamblia* antigen being used for detecting anti-giardial antibodies in sera of 60 human subjects. Titers in some of these subjects were 1 : 80–1 : 2560, whereas titers in some subjects were negative button to 1 : 20. The results indicated that *Giardia lamblia*, an intestinal parasite, induced a systemic antibody response and the indirect haemagglutination test for anti-*Giardia lamblia* antibodies is a simple specific and reproducible system which may be useful in epidemiologic and immunologic studies of giardiasis. The specificity of the anti-bodies was demonstrated by the ability of live *Giardia lamblia* trophozoites, but not *Entamoeba histolytica* to absorb the antibody activity.

Keywords. Haemagglutination; giardiasis.

Introduction

Giardia lamblia is now recognised as a frequent gastrointestinal pathogen all over the world (Morbid Mortal Weekly Report—Centers for Diseases Control, 1978). The parasitological diagnosis of giardiasis involves repeated examination of faeces and in certain cases examination of duodenal aspirate or jejunal biopsy (Petersen, 1972; Yardley *et al.*, 1972). The application of these methods in large scale epidemiological studies is not practical. Detection of circulating antibody to *G. lamblia* has recently been demonstrated both by indirect immunofluorescence (IFA) (Ridley and Ridley, 1976) and an enzyme linked immunosorbent assay (ELISA) (Smith *et al.*, 1981). These methods however are not always suitable for epidemiological studies, since IFA technique is subject to observer variation and requires expensive equipment and for the ELISA, live axenic giardia cultures are needed. This study describes a simple test involving indirect haemagglutination (IHA) as the basis for the detection of circulating antibody to *G. lamblia* both in human subjects as well as in rabbits immunised with whole giardial antigen.

Materials and methods

Sera

Human: Blood was collected from 60 subjects, including both symptomatic and nonsymptomatic human subjects, and sera were prepared.

Abbreviations used: IFA, Immunofluorescence; ELISA, enzyme linked immunoabsorbent assay; IHA, indirect haemagglutination; PBS, phosphate buffered saline; SRBC, sheep red blood cells.

Rabbit: Blood was also obtained from 4 rabbits, each immunised with 3 injections of whole homogenate of giardia antigen at 3 weekly intervals, and from 4 control rabbits which were similarly injected with Freund's adjuvant only without any giardia antigen.

Giardia antigen: Giardia antigen, was prepared from axenically maintained portland strain of *G. lamblia* obtained from Dr. Louis Diamond, National Institute of Health, USA. Organisms were cultured in a modified Diamond's TPS-1 medium supplemented with 3% vitamins and 10% bovine serum (Diamond, 1968; Meyer, 1976).

   Actively growing, 3–4 days old *G. lamblia* organisms were dislodged from the walls of the borosilicate (16×125 mm) glass tube by immersing in ice water for 5 min and were then centrifuged ($250\ g$ for 10 min). The harvested cells were used as antigen.

Antigen for IFA assay: The *G. lamblia* cells harvested were diluted with 50 mM phosphate buffered saline (PBS) so that 1 drop contained between 1000–2000 organisms per $100 \times$ microscopic field. One drop of this suspension was placed in the centre of each glass slide which was then dried at room temperature. The slides, when just moist, were immersed in 90% alcohol for 10 min for fixation. These slides were then usded in IFA assay (Visweswara *et al.*, 1980) using the human serum and fluoreceinisothiocyanate conjugated rabbit anti-human globulin pre-pared in this laboratory. Serially diluted human sera samples were used. The same sera dilutions were also evaluated by the IHA test described subsequently.

Antigen for IHA test: The harvested *G. lamblia* cells were suspended in 3–5 ml of 50 mM PBS (pH 7·2) containing 1% Triton X-100. The cells were homogenized using Potter Elvejhem homogenizer. The homogenates were then centrifuged at $9220\ g$ for 30 min (in a Sorval RC 5-SS34 rotor). After centrifugation, the supernatant containing the antigen was dialysed against 50 mM PBS at 4°C overnight. On completion of dialysis, protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. The antigen preparation with a protein concentration adjusted to 2 mg/ml was then used for coating 50% formalised tanned sheep red blood cells (SRBC). Another batch of antigen was prepared in the above way but without the addition of Triton X-100 (non-tritonated).

IHA assay: Using the above antigen coated SRBC, haemagglutination assays were performed on microtiter plate (Kessel *et al.*, 1965). Both human and rabbit sera were tested in serial dilutions from 1/20 to 1/10240. With rabbit sera, two sets of haemagglutination tests were done, one using tritonated antigen and the other prepared without triton. Three to four repeat IHA tests were performed on the same sera samples using different batches of antigen, freshly prepared as well as stored in liquid nitrogen.

Determination of antibody specificity: Antibody specificity of the indirect haemag-glutination assay was evaluated by preabsorbing human and rabbit antibody positive sera alongwith control negative sera with equivalent numbers of trophozoites of *G. lamblia*. In separate experiments, 1 ml from each of the above sera was incubated for 24 h at 4°C with 1 ml containing 59.2×10^5 washed *G.*

lamblia organisms and 23.9×10^5/ml of washed *Entamoeba histolytica* organisms. The axenic *E. histolytica* was cultured in Diamond's TY1-S-33 medium (Diamond, 1968). On completion of incubation, the sera were centrifuged at 660 g for 20 min and used for IHA assay using tritonated giardial antigen coated SRBC.

Results

Rabbit sera

It was noted that non-tritonated giardia antigen had no detectable haemagglutination activity, whereas the tritonated fraction gave a positive haemagglutination reaction with rabbit sera immunised with *G. lamblia*. Sera from control animal showed no haemagglutination (figure 1).
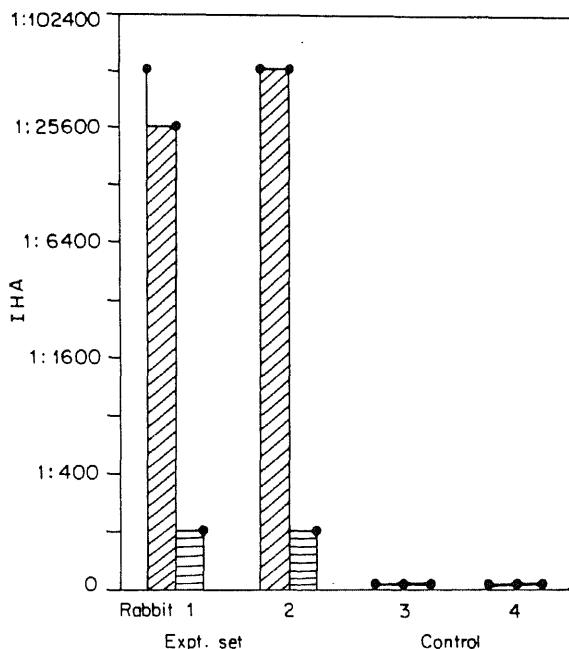
Figure 1. IHA titres of the rabbit antigiardia antiserum. IHA assay of this rabbit anti-giardia antisera was done with two types of giardial antigen. (i) Tritonated antigen (▨). (ii) Nontritonated antigen *i.e.* soluble supernatant of homogenized giardia cells (▤).

All the sera collected from 60 human subjects were subjected to indirect haemagglutination assay test using tritonated giardial antigen coated SRBC. In 38 samples the indirect haemagglutination titres were found to be 1/80 and above and when the stool of these subjects were tested it was found that in 3 repeated stool examinations *G. lamblia* could be detected. In 18 cases, the sera did not show any haemagglutination activity (titre was very low, 1 : 20, 1 : 40). The stools of these subjects did not show the presence of *G. lamblia*. However, 4 of the 60 human subjects gave a titre of 1 : 80 or higher but their stool examination gave a negative

result. However, the presence of G. *lamblia* infection in these 4 patients can not be excluded since neither their duodenal aspirate nor jejunal biopsies were examined (figure 2).
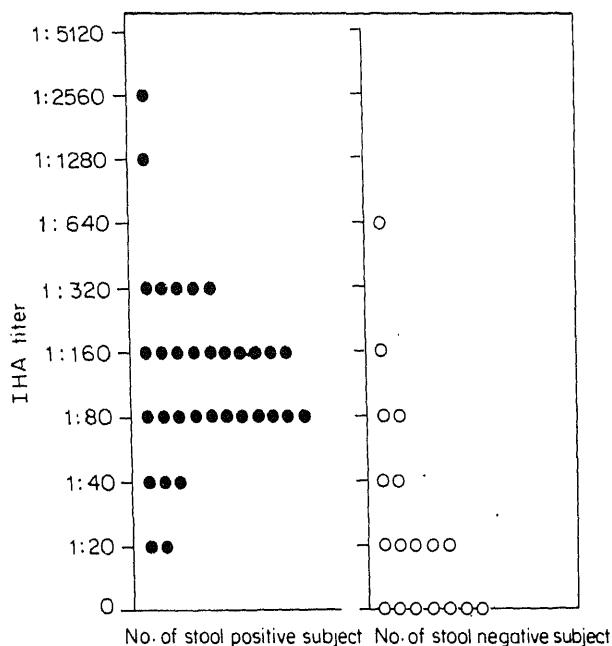
Figure 2. IHA titres and the stool reaction of 60 subjects.

Specificity of the anti-G. *lamblia* antibody was evaluated by absorbing the sera (figure 3) in two separate sets with E. *histolytica* and G. *lamblia*. The E. *histolytica* absorption did not reduce the indirect haemagglutination titre of the sera, but absorption with G. *lamblia* reduced the indirect haemagglutination titre significantly.

To evaluate the reproducibility of IHA, the same sera were tested by IHA using 3 different batches of antigens and the resultant IHA titres were similar.

Correlation of IHA and IFA

The human sera tested for IHA were also titred for antibody to G. *lamblia* using an IFA technique. There was a good correlation (figure 4) between IHA and IFA titres. The correlation coefficient between IHA amd IFA is 0·5601197.

Discussion

A simple diagnostic tool for detecting giardiasis is needed for epidemiological studies. Epidemiological study involving parasitological evaluation of stool can be difficult. The existing methods of IFA and ELISA for determining antigiardial antibody are too sophisticated and expensive to be within the reach of ordinary
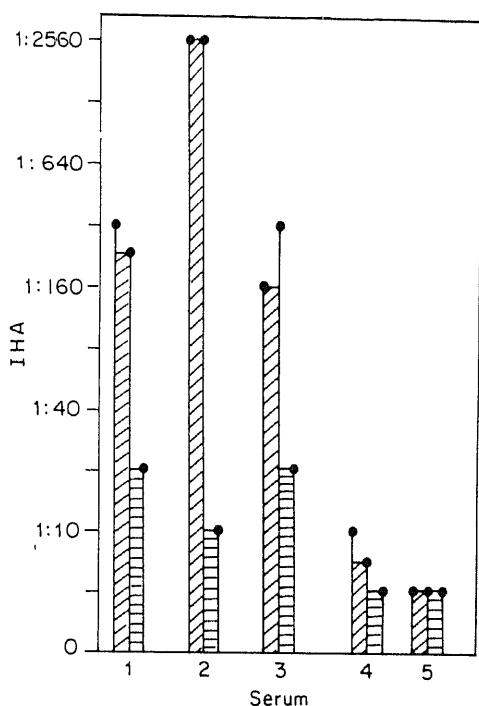
Figure 3. Efficacy of sera of IHA positive and IHA negative subjects after adsorption with *G. lamblia* and *E. histolytica* cells. (●), IHA titre without absorption; (▨), IHA titre after absorption with *E. histolytica* cells; (☰), IHA titre after absorption with *G. lamblia* cells.
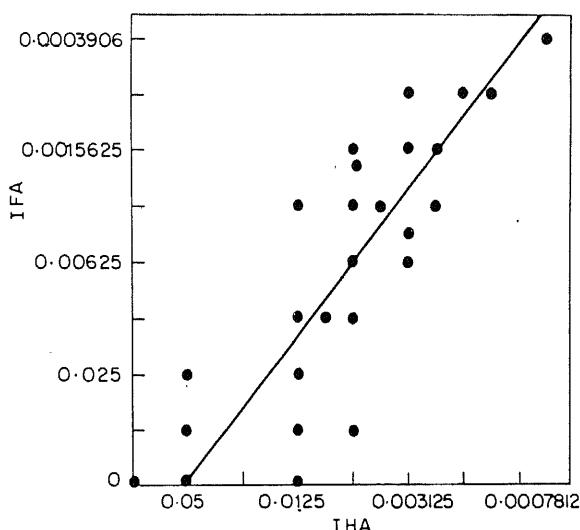
Figure 4. Correlation plot of indirect haemagglutination assay and indirect fluorescence assay titres of 60 human subjects. Equal dilutions of these human sera samples were used in both the assays. The IHA and IFA titres are indicated in terms of dilutions of the sera samples of these subjects. (●), Point of coincidence of the result by IHA and IFA assay methods.

laboratories in developing countries. This study undertaken to devise a simple diagnostic method for detecting giardiasis has achieved the following:

(i) An indirect haemagglutination test for the detection of antibodies to *G. lamblia* has been developed.

(ii) Sera from rabbits immunised with giardial antigen have confirmed the validity of this haemagglutination test.

(iii) The pattern of antigiardial antibody activity in human subjects both positive or otherwise for *G. lamblia* in stool has been established.

The IHA developed is quantitative and reproducible. It is simple and requires only a small amount of serum. The antigen once prepared can be stored in liquid nitrogen and used as and when needed.

Employing the IHA test, it has been shown that none of the patients with a positive stool reaction had a negative IHA titre and majority of them had titres above 1/80. However, 4 of the control patients with negative stool reaction had titres above 1/80. However, in these cases the presence of giardia in the duodenum can not be excluded. High titres in these patients could be a reflection of an infection in the near past. The haemagglutinating antibody is probably of the IgG type, since antibodies of other types are not expected to have haemagglutinating activity.

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Cloning of Y derived DNA sequences of bovine origin in *Escherichia coli*

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Abstract. We have constructed a partial library of Y chromosome derived DNA sequences of bovine origin in *Escherichia coli*. That, the recombinants are Y derived and Y specific was ascertained by differential colony hybridization using male and female DNA probes. Out of 1000 recombinants analysed, 17 were found to be Y derived as well as Y specific and were of repetitive nature. Restriction analysis revealed that most of them had short DNA inserts.

Introduction

The sexually differentiated state in mammals is determined by 'Y' chromosome and it appears that sex determining genes of Y chromosome behave as dominant genetic trait. Role of histocompatibility Y antigen in male differentiation is envisioned although its precise function is not yet clear (Silver and Wachtel, 1977; Wachtel, 1977; McCarey and Abbot, 1979). The first step towards analysing Y encoded genes involved in sexual differentiation would be to isolate and clone the chromosomal DNA. A variety of approaches have been used for cloning Y encoded sequences of human, murine and reptile origin (Kunkel *et al.*, 1976; Cooke, 1976; Jones and Singh, 1981; Lamer and Palmer, 1984). Repetitive as well as single copy DNA sequences specific to human male DNA have been identified (Kunkel *et al.*, 1976; Cooke, 1976; Bishop *et al.*, 1983) and their localization on Y chromosome has been assigned. Y derived sequences of human origin have provided powerful analytical tools to probe directly the structure of Y chromosome (Cooke and Mackay, 1978; Kunkel, *et al.*, 1979; Cook, 1982; Bostock *et al.*, 1978) and also have been used for fetal sex determination (Gosden *et al.*, 1982; Gosden *et al.*, 1984). Relatively little information is available on Y specific determinants in cattle. Cross reactivity of human Y sequences with cattle has not been reported. In fact the unique sequences of human origin, when tested, did not hybridize with cattle DNA (J. Weissenbach, unpublished results). In this communication we report the construction of a partial library of bovine Y derived sequences.

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Materials and methods

All restriction endonucleases, T_4 DNA ligase, bacterial alkaline phosphatase, DNA polymerase and DNase I were from Bethesda Research Laboratory, Gaithersburg, Maryland, USA. Protease and Ribonuclease A were from Sigma Chemical Corporation, USA. Ampicillin, tetracycline and chloramphenicol were bought from Sigma Chemical Co., St. Louis, Missouri, USA. [α-^{32}P]-dCTP and [α-^{32}P]-dTTP (800 Ci/mmol) were from new England Nuclear.

Isolation of DNA

DNA was isolated from bovine leucocytes according to method described (Vergnaud *et al.*, 1984). For preparation of donor (hybrid) DNA, Sau III A digested male DNA (5 μg) was hybridized in phosphate buffer with 500 μg of sheared female DNA (average size, 500 bp) to a Cot value of 1000 followed by fractionation by chromotography on hydroxylapatite as described (Eden *et al.*, 1978). Plasmid DNAs were prepared as described by Clewell and Helinski (1972).

Construction of recombinant plasmids carrying Y derived sequences

Male-female hybrid DNA was cloned into *Escherichia coli*, strain HB 101 using *Bam*H1 site in plasmid pBR 322 (Mandel and Higa, 1970). Recombinants scored on the basis of ampicillin resistance and tetracycline sensitivity were further analysed by differential colony hybridization technique.

Radiolabelling of bovine DNAs

DNA from male and female cattle (2 μg) was radiolabelled with [α-^{32}P]-dCTP, [α-^{32}P]-dTTP by nick translation procedure (Rigby *et al.*, 1977). On an average, specific activity obtained was 1–2×10^8 cpm/μg of DNA.

Colony hybridization

Recombinant colonies grown on nitrocellulose membrane filters were lysed by treatment with 0·5 M NaOH and colony hybridization was done essentially as described by Maniatis *et al.* (1982).

Results and discussions

Although Y chromosome specific repetitive DNA sequences of human origin were identified by analysis of male-female DNA restriction digests on agarose gel (Cooke and Mackay, 1978), this was not found to be useful for cattle DNA. Comparison of male DNA restriction digests with female DNA restriction digests for restriction endonucleases *Eco*RI, *Hae*III and *Hind*III did not reveal any specific bands on male DNA digest (figure 1). Instead, strong bands of varying intensity were visible over background smear, migrating with same mobility in male as well as female DNA digests. This may suggest that organization of Y derived repetitive sequences for cattle may not be the same as that of human.
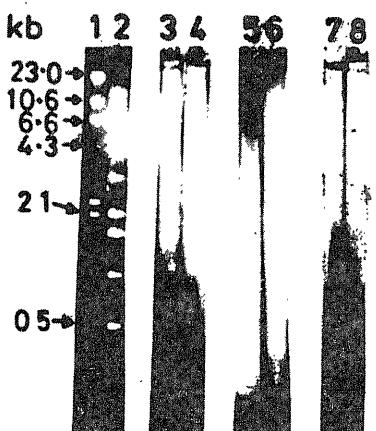
Figure 1. Restriction analysis of male and female cattle DNAs. 10 μg of DNA was digested to completion with restriction endonucleases *Eco*RI, *Hae*III and *Hind*III. DNA fragments were separated on 1% agarose gel and visualised after staining with ethidium bromide. Lanes 1 and 2, standard DNA markers; 3 and 4, *Eco*RI digested male and female DNAs; 5 and 6, *Hae*III digested male and female DNAs; 7 and 8, *Hind*III digested male and female DNAs.

Construction of partial library of Y derived DNA sequences in E. coli

To construct Y chromosome derived DNA library, the strategy used by us was similar to that used by Lamer and Palmer (1984). DNA from male cattle was digested to completion with restriction endonuclease *Sau*3A, while female DNA was sheared to an average size of 500 bp fragments. Male DNA was hybridized with large excess of female DNA (see 'materials and methods') to a Cot of 1000. Amongst 3 different kinds of hybrid molecules expected (*i.e.*, male-male, male-female and female-female), male-male hybrids should predominently represent Y derived sequences with *Sau*3A sticky ends, while sequences that are common between male and female DNAs will be present in the other two kinds of hybrid molecules. The hybrid DNA, purified free of single stranded DNA by hydroxyl apatite chromatography (figure 2) was used for cloning.

Restriction endonuclease *Sau*3A recognises the base sequence GATC which is common to the internal 4 bases of the sequences recognised .by *Bam*H1 (G$^{\downarrow}$GATCC). Thus *Sau*3A derived DNA fragments can be cloned into *Bam*H1 site. Thus male-male hybrid molecules enriched for Y specific sequences and having *Sau*3A sticky ends were cloned into *E. coli* using *Bam*H1 site of plasmid pBR 322. From 10 μg of total hybrid DNA 1000 recombinants were obtained which were subjected to further analysis.

Identification of male specific recombinants

The strategy employed was mainly based on the assumption that there are sequences present on the Y chromosome that are not present in the female DNA. In order to ascertain that the partial library constructed by us has Y derived as well
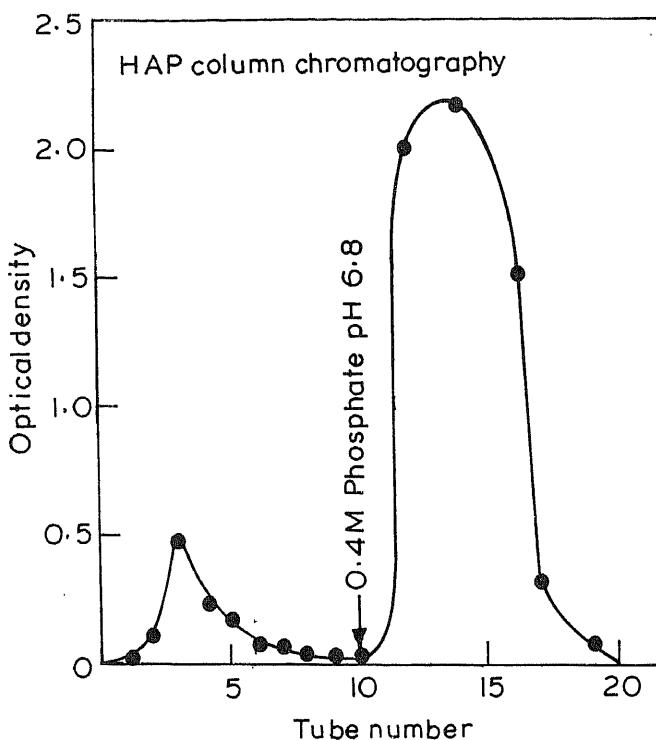
Figure 2. Hydroxylapatite column chromatography of hybrid DNA. Hybrid DNA in 0.12 M phosphate buffer, pH 6.8 was passed through HAP column (2 ml bed volume) equilibriated in the same buffer at 60°C. The column was washed with several volumes of 0.12 M phosphate buffer and the double stranded DNA was eluted from the column with 0.4 M phosphate buffer (fraction size, 0.5 ml).

as Y specific sequences, a differential colony hybridization experiment was carried out. Recombinants were grown in duplicate on nitrocellulose membrane filters in the presence of ampicillin and hybridized separately with male as well as female DNA probes of very high specific activity (see 'materials and methods' and legend to figure 3). The specific activity for both the probes was of the same order and same number of counts were used in hybridization. On the basis of autoradiographic signal the recombinants were grouped into 3 categories: (i) Those that gave strong hybridization signal of same intensity with both the probes, (ii) those which gave very strong hybridization signal with male DNA probe in comparison with female DNA probe and (iii) those with very weak signal and indistinguishable between male and female DNA probes. Out of 1000 colonies analysed, 17 fell into category II, where a stronger signal was observed with male DNA in comparison with female DNA probe. This is indicative of the presence of Y derived and Y specific determinants in them. Y derived repetitive sequences of human are also reported to be present on X chromosome (Cooke and Mackay 1978) and the first category of recombinants may be of this type. Weak hybridization signal observed with the remaining colonies may be due to single copy sequences present in them. Although hybridization of male and female cattle DNA was carried out at a Cot of

1000, where most of the single copy and repetitive sequences were collected in 3 different kinds of hybrid molecules, only highly repetitive sequences were identifiable on the basis of colony hybridization. The probes used in differential colony hybridization experiments were nick translated male and female total DNAs, of same specific activity (see legend to figure 3) and within themselves the sequences of repetitive nature should have higher specific activity in comparison to single copy sequences. The conditions used for hybridization as well as stringent washings and exposure time allowed detection of recombinants carrying highly repetitive sequences (male specific) (figure 3).
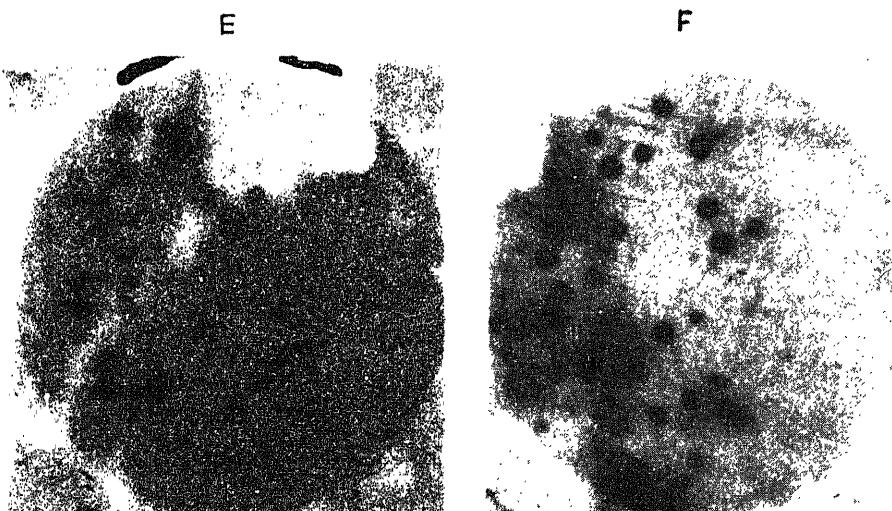
Figure 3. Identification of recombinants by colony hybridization. Recombinants were grown in duplicate on membrane filters (0.45 mμ) at 37°C for 18 h, lysed with 0.5 M NaOH and the denatured DNA was fixed after baking the filters at 80°C for 2 h. Each filter was hybridized separately with 5×10^6 cpm of ^{32}P labelled, nick translated total male or female DNAs (materials and methods). E and F represent hybridization profile with male and female DNA probes, respectively. Specific hybridization signal with male probe is indicated by arrows.

Recombinants of the second catagory were analysed for the presence of DNA insert. Analysis of DNA fragments after *Eco*RI as well as *Hin*CII digestion revealed that inserts were in the range of 200–800 bp (data not shown). Similarly, the nature of inserts as determined by southern blot hybridization experiments revealed coexistence of Y specific and non Y determinants on the cloned DNA (published elsewhere). Such observations are reported in the case of 3·4 kb *Hae*III fragment of human origin (Cooke, 1976). Screening of more recombinants from expanded library as well as their characterization are in progress.

Acknowledgements

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Effect of magnesium deficiency on the metabolism of glycosamino-glycans in rats

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Abstract. Magnesium deficiency in rats has significant effect on the concentration of different glycosaminoglycans in the tissues, the nature of the change being different in different tissues. Total glycosaminoglycans, chondroitin-4-sulphate + chondroitin-6-sulphate and dermatan sulphate increased in the aorta while hyaluronic acid, heparan sulphate and heparin decreased. In the liver, total glycosaminoglycans, hyaluronic acid, chondroitin-4-sulphate + 6-sulphate and heparin decreased while total glycosamino-glycans and all the glycosaminoglycan fractions increased in the heart. In the kidney, total glycosaminoglycans showed no significant alteration, hyaluronic acid and heparin decreased while chondroitin-4-sulphate + 6-sulphate increased. Activity of biosynthetic enzymes *viz.* glucosamine-6-phosphate isomerase and UDPG-dehydrogenase showed decrease in the liver. The concentration of 3'-phosphoadenosine 5'-phosphosulphate, activity of sulphate activating system and sulphotransferase were also similarly altered in the liver in magnesium deficiency.

Keywords. Magnesium deficiency; hyaluronic acid; heparan sulphate; chondroitin-4-sulphate; chondroitin-6-sulphate; dermatan sulphate; heparin; biosynthetic enzyme; sulphation.

Introduction

Magnesium is essentially an intracellular cation with important biochemical and physiological functions which include participation in energy metabolism, membrane permeability, electrolyte transport, nucleic acid metabolism, displacing Ca^{2+} from membrane receptors and binding sites of enzymes (Ebel and Gunther, 1983), developing of action potential (Bara *et al.*, 1983), hydroxylation of vitamin D (Traba *et al.*, 1983), transphosphorylation reactions mediated by insulin (Greengard, 1978; Ichiara and Murd, 1979), and coagulation processes (Sneddon, 1972). Some data are also available on Mg-lipid interrelationship (Rayssiguier 1983).

It is known that the macromolecular components of inter-cellular matrix *viz.* proteoglycans (pg), glycoproteins, collagen, elastin etc. play, among other

functions, a role in cell to cell interactions and in the transport of substances from and into the cell. Among these macromolecular components, pg are the most important, some of which are also known to be components of cell membranes. Since Mg^{2+} deficiency among other things, affects membrane permeability and transport of substances, it is necessary to know whether the metabolism of glycosaminoglycans (gg) (most of which are present in the tissues as pg) is affected in Mg^{2+} deficiency. No data seem to be available in this respect. In view of this, the effect of Mg^{2+} deficiency on the metabolism of gg has now been studied using rats as experimental animals.

Materials and methods

Male albino rats (Sprague-Dawley strain, weighing 80–100 g) were divided into the following groups with 15 rats in each each group: (i) Control group, and (ii) experimental Mg^{2+} deficient group.

The diet had the following composition (g/100 g diet): Dextrose, 69·0; Casein (vitamin and fat free), 18·0; groundnut oil, 8·0; salt mixture, 4·0; vitamin mixture, 1·0.

Wesson's salt mixture without Mg^{2+} was used (Oser, 1965) $Zncl_2$ and $CoCl_2$. $6H_2O$ were also added to the diet at a concentration of 15·0 and 0·15 mg/kg diet, respectively. The Mg content of the diet was determined by analysis and $MgSO_4$ (AR : BDH) was added to the diet to give a dietary Mg level of 5 mg/100 g diet in the deficient group and 100 mg/100 g diet in the control group. All the chemicals used for the salt mixture were of analytical grade. Vitamin mixture used had the same composition as described earlier (Jayakumari and Kurup, 1979). The rats of group 1 were pairfed controls of those of group 2. The diet consumption was adjusted to be the same in the two groups. Deionized water after distillation was available to the rats adlibitum. The rats were housed individually in polypropylene cages in rooms maintained at 25°C. The duration of the experiment was 60 days. At the end of this period, the animals were deprived of food overnight, stunned by a blow at the back of the neck and killed by decapitation. Blood and tissues were removed to ice cold containers for various estimations.

Estimation of gg in the tissues

Dry defatted tissue was subjected to digestion with papain [crystalline papain (Sigma), 1/3 the dry weight of the tissue] (Laurent, 1960). The papain digest was deproteinised with trichloroacetic acid (TCA) (final concentration of TCA, 10% v/v) and the supernatant dialysed till free of TCA. Total gg was precipitated from the solution by the addition of 4–5 volumes of 95% v/v ethanol containing 1·2% (w/v) potassium acetate. The precipitate collected by centrifugation was dissolved in a known volume of water. An aliquot of this solution was used to determine total gg by estimating uronic acid content by the modified procedure of Bitter and Muir (1982). Another aliquot of the gg solution (approximately 100 μg uronic acid) was digested with testicular hyaluronidase (Sigma Chemicals, 100 units) in 0·25 M acetate buffer pH 5·3 containing 0·75 M NaCl for 16 h, under a layer of toluene. The undigested gg [containing heparan sulphate (HS), dermatan

sulphate (DS) and heparin (H)] was precipitated from the solution by 95% ethanol (v/v) containing potassium acetate as described above. The precipitate collected after centrifugation was washed with the ethanol solution, dissolved in a known volume of water and uronic acid estimated.

Another aliquot of the gg solution (approximately 100 μg uronic acid) was digested with chondroitinase ABC (Sigma Chemicals, 0·02 unit) in Tris-HCl buffer (0·25 M), pH 8·0 containing bovine serum albumin, sodium acetate and sodium chloride (50 mg, 2·4 g and 1·4 g, respectively/100 ml of the solution) for 16 h under toluene. The undigested gg [hyaluronic acid (HA), HS and H] was precipitated with 95% (v/v) ethanol as described above. The precipitate was dissolved in a known volume of water and the solution passed through a column of Dowex 1×2 (Cl⁻ form), 0·5×5 cm. The column was washed with water, eluted successively with 0·25 M, 1·7 M and 3·5 M NaCl solution. These eluates contained HA, HS and H, respectively. The uronic acid in each eluate was estimated as before. From these, the amount of HA, HS, chondroitin-4-sulphate (Ch-4S) + Chondroitin-6-sulphate (Ch-6S), DS and H were obtained.

Estimation of enzyme activities

The activity of D-glucosamine-6-phosphate isomerase [glutamine forming (EC 2·6·1·16) was estimated in the tissue according to the procedure of Pogell and Gryder (1957)]. Activity of UDPG-dehydrogenase (UDP-glucose: NAD oxidoreductase) (EC 1·1·1·22) activity was determined by the method of Strominger *et al.* (1957).

Sulphate metabolism

The concentration of 3′ phosphoadenosine 5′ -phosphosulphate (PAPS), and the activity of sulphate activating system (sulphate adenyl transferase) (EC 2·7·7·4) and adenyl sulphate kinase (EC 2·7·1·25) and that of aryl sulphotransferase (EC 2·8·2·1) of liver were estimated by the method of Van Kempen and Jansen (1973) using methyl umbelliferone. Details of the procedure have been described earlier (Sudhakran and Kurup, 1974).

Estimation of Mg^{2+} in the serum and tissues was carried out by atomic absorption spectrophotometry. The tissues were first ashed and then dissolved in dilute HCl. Glutathione in the erythrocytes was estimated according to the procedure of Lazarow *et al.* (1955). Protein in the enzyme extract was determined after TCA precipitation by the method of Lowry *et al.* (1972).

Statistical analysis was carried out by Student's '*t*' test (Bennet and Franklin, 1967).

Results and discussion

Magnesium deficient rats consumed less food (average value during the duration of the experiment being 8·0±1·0 g) and had lower weight gain. Liver and spleen weights were higher in these rats (table 1).

The concentration of Mg^{2+} in the serum in the rats of group 1 and 2 was 1·72±0·04 and 0·44±0·1 mEq/litre, respectively. The Mg^{2+} concentration in the

Table 1.   Weight gain and organ weight in magnesium deficiency.

Group	Weight gain (g/rat/60 days)	Liver weight (g)	Spleen weight (g)
Control	$58 \cdot 0 \pm 1 \cdot 2$	$4 \cdot 87 \pm 0 \cdot 1$	$0 \cdot 86 \pm 0 \cdot 01$
Mg deficient	$37 \cdot 4 \pm 0 \cdot 64^a$	$6 \cdot 17 \pm 0 \cdot 12^a$	$1 \cdot 55 \pm 0 \cdot 03^a$

Values are the mean $\pm$ SEM for 15 rats. Group 2 has been compared with group 1. $^a P < 0 \cdot 01$.

liver in these groups was $18 \cdot 2 \pm 0 \cdot 49$ and $14 \cdot 5 \pm 0 \cdot 36$ mEq/kg wet tissue. Concentration of glutathione in the erythrocytes in groups 1 and 2 were $372 \cdot 5 \pm 10 \cdot 8$ and $292 \pm 3 \cdot 46$ μ mol/100 ml packed cells, respectively. The lower value for glutathione in the erythrocytes in Mg^{2+} deficient rats is in agreement with the results reported by Jeng *et al.* (1982).

Changes in the concentration of gg in different tissues (table 2)

Liver: Total gg, HA, 4S + 6S and H decreased in the liver in the Mg^{2+} deficient rats while HS and DS showed no significant alteration. The activity of some enzymes involved in the biosynthesis of precursors of gg (glucosamine-6-phosphate isomerase and UDPG dehydrogenase) showed a significant decrease (table 3). These enzymes catalyse rate limiting steps in the biosynthetic pathway of gg, glucosamine-6-phosphate isomerase being inhibited by feed back inhibition by UDP-N-acetyl glucosamine and UDPG dehydrogenase by UDP-xylose. Therefore the decrease in the activity of these enzymes may result in decreased biosynthesis of gg in the liver.

Sulphate metabolism in the liver (table 4): The concentration of PAPS, activity of sulphate activating system (which includes sulphate adenyl transferase and adenyl sulphate kinase) and that of sulphotransferase decreased in the liver in the Mg^{2+} deficient rats. The decrease in the concentration of PAPS is due to the decrease in the activity of sulphate activating system which generates it. Thus sulphate metabolism is decreased in Mg^{2+} deficiency. The decrease in the concentration of some of the sulphated gg in the liver may also be due to decreased sulphation. Thus the metabolism of gg in the liver is significantly altered in Mg^{2+} deficiency.

Aorta

The concentration of total gg and that of 4S + 6S and DS increased in the aorta in the Mg^{2+} deficient rats while HA, HS and H decreased. The aorta is particularly important in view of the high concentration of gg present. The gg are believed to play a role in the transport and accumulation of lipids in the arterial tissue. The presence of negatively charged carboxyl and sulphate groups in the gg enables these molecules to bind those bearing positive charges. Complex formation between lipoproteins [very low density lipoproteins (VLDL) and low density lipoproteins (LDL)] and gg has been shown in a number of *in vitro* studies (Burstein and Samaille, 1955; Bernfield *et al.*, 1960; Cornwell and Kruger, 1961; Iverius, 1972;

Table 2. Concentration of gg in different tissues in magnesium deficiency.

Tissue	Group	Total gg	HA	HS	4S+6S	DS	H
				(μg/g dry defatted tissue)			
Aorta	1	6180±150	640·0±16	1560±37·4	2430·0±58·3	890·0±23·1	540·0±14
	2	6767±203[a]	385·3±8·7[a]	1380±34·5[a]	3406·4±92·0[a]	1171·7±26·9[a]	290·0±7[a]
Liver	1	1072±25	150·0±4	230±5	320·0±8·0	140·0±3	200·0±5
	2	928±22[a]	130·0±3[a]	230±5	278·1±7·2[a]	143·6±3·3	130·0±4[a]
Heart	1	1540±35	340·0±9	360±9	480·0±12	160·0±3·5	140·0±4·1
	2	2440±60[a]	600·0±15[a]	530±12[a]	784·9±20·4[a]	193·2±5·4[a]	268·3±7[a]
Kidney	1	1720±50	335·0±8·0	330±8	555·0±12·8	223·0±5·4	227·0±6
	2	1705±45	287·9±7·8[a]	356±9	606·3±14·6[a]	209·1±5·5[a]	186·0±5[a]

Values are the mean ± SEM for 6 rats. Group 2 (magnesium deficient) has been compared with group 1 (controls). [a] $P<0.01$. [b] Between 0·01 and 0·05.

Table 3. Activity of glucosamine-6-phosphate isomerase (glutamine forming) and UDPG dehydrogenase in liver of magnesium deficient rats.

Group	Glucosamine-6-phosphate isomerase (glutamine forming) (μ mol of hexosamine/h/ g protein)	UDPG dehydrogenase (Units*/g protein)
Control	36·0 ± 0·9	2280·0 ± 52.4
Magnesium deficient	22·4 ± 0·6^a	1865·5 ± 48·5^a

* The amount of enzyme required to give an increase of 0·001 in absorbance/g protein.
^{a} Same notations as in table 2.

Table 4. Sulphate metabolism in the liver of magnesium deficient rats.

Groups	Concentration of PAPS	Sulphate activating system	Sulpho- transferase
	(μ mol of methyl umbelliferone sulphate formed/h/g protein)		
Control	165·0 ± 4·1	27·9 ± 0·7	22·0 ± 0·6
Magnesium deficient	138·3 ± 3·2^a	15·3 ± 0·4^a	34·8 ± 0·9^a

^{a} Same notations as in table 2.

Srinivasan *et al.*, 1970). It has also been shown that gg lipoprotein complex can be extracted from the arterial tissue (Srinivasan *et al.*, 1972). The increase in the chondroitin sulphate isomers in the aorta in Mg^{2+} deficient rats along with increase in the VLDL + LDL cholesterol reported earlier (Jaya, P. and Kurup, P. A., unpublished results) may result in increased complex formation with resultant increase in the accumulation of lipids in this tissue. The results are in agreement with increased concentration of cholesterol observed in the aorta in Mg^{2+} deficient rats (Vitale *et al.*, 1957; Jaya, P. and Kurup, P. A., unpublished results).

Heart and Kidney

Total uronic acid and the concentration of different gg increased in the heart of rats fed Mg^{2+} deficient diet when compared to pairfed control rats. The concentration of total gg showed no significant alteration in the kidney in the Mg^{2+} deficient rats but HA and H decreased while 4S + 6S increased. There was no significant alteration in HS or DS.

Judging from the results of changes in gg in the aorta and heart now observed and the changes in the lipids in the serum and the tissues (Jaya, P. and Kurup, P. A., unpublished results), Mg^{2+} deficiency is seen to produce changes which are generally similar to those observed in atheromatous rats (Vijayakumar and Kurup,

1975; Vijayakumar *et al.*, 1975) and also in rats in which myocardial infarction is induced by isoproterenol (Mathew *et al.*, 1982). Decreased concentration of Mg^{2+} has been reported in the serum (Brown *et al.*, 1958) of patients of myocardial infarction. In endomyocardial fibrosis also, which is prevalent in Kerala, significantly lower concentrations of Mg^{2+} has been reported in the heart and the fibrotic tissues has been found to contain high concentrations of glycosaminoglycans (Valiathan, M. S., unpublished reports). Therefore the finding that Mg^{2+} deficiency induced higher concentration of gg in the heart is of significance.

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